

POLYAMINES, growth regulators and tumor markers

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Lund 1984



POLYAMINES:
GROWTH REGULATORS AND
TUMOR MARKERS

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LUND 1984

*"A thing of beauty
is a joy for ever"*

To
my husband's relief

The present thesis is based on the following papers:

- I. Oredsson S., Anehus S. & Heby O.
Irreversible Inhibition of the Early Increase in Ornithine Decarboxylase Activity Following Growth Stimulation is Required to Block Ehrlich Ascites Tumor Cell Proliferation in Culture.
Biochem. Biophys. Res. Commun. 94:151-158, 1980.
- II. Anehus S., Pohjanpelto P., Baldetorp B., Långström E. & Heby O.
Polyamine Starvation Prolongs the S and G2 Phases of Polyamine-Dependent (Arginase-Deficient) CHO Cells.
Mol. Cell. Biol. 4, May 1984 (in press).
- III. Anehus S., Emanuelsson H., Persson L., Sundler F., Scheffler I.E. & Heby O.
Localization of Ornithine Decarboxylase in Mutant CHO Cells That Overproduce the Enzyme. Differences Between the Intracellular Distribution of Monospecific Ornithine Decarboxylase Antibodies and Radiolabeled α -Difluoromethylornithine.
Submitted for publication.
- IV. Anehus S., Bengtsson G., Andersson G., Carlsson G., Hafström L., Yngner T. & Heby O.
Urinary Putrescine and Plasma Lactate Dehydrogenase as Markers of Experimental Adenocarcinoma Growth.
Eur. J. Cancer 17:511-518, 1981.
- V. Anehus S., Albinsson A., Andersson G. & Heby O.
Urinary Polyamine Excretion During Growth and Regression of 7,12-Dimethylbenz(a)anthracene-Induced Rat Mammary Carcinoma.
J. Natl. Cancer Inst. 67:1265-1268, 1981.
- VI. Anehus S., Yngner T., Engelbrecht C., Hafström L. & Heby O.
Urinary Polyamine Excretion as Related to Cell Death and Cell Proliferation Induced by Carbon Tetrachloride Intoxication.
Exp. Mol. Pathol. 38:255-263, 1983.

These papers will be referred to in the text by their Roman numerals.



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1. ABBREVIATIONS

CHO cells	Chinese hamster ovary cells
CHO-A7 cells	Chinese hamster ovary cells deficient in arginase
DFMO; ^3H -DFMO	α -Difluoromethylornithine; α -(5- ^3H)difluoromethylornithine
DMBA	7,12-Dimethylbenz(a)anthracene
ELD	Ehrlich Lettré Diploid ascites tumor cells
MGBG	Methylglyoxal-bis(guanyldrazone)
MO	α -Methylornithine
NG-W1	N-Methyl-N'-nitro-N-nitrosoguanidine-induced, transplantable adenocarcinoma
ODC	Ornithine decarboxylase
SAMDC	S-Adenosylmethionine decarboxylase

2. BACKGROUND

2.1 POLYAMINE STRUCTURE

The polyamines, putrescine, spermidine and spermine, are normal constituents of pro- and eukaryotic cells and their viruses (1-3). They are low-molecular, aliphatic amines with all their amino and imino groups positively charged at a physiological pH (Fig. 1). The polyamines may neutralize the negatively charged phosphate groups of the nucleic acids (2, 4-6). As a result of this electrostatic binding, they stabilize the nucleic acids against denaturation and shearing (7, 8).

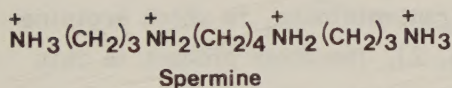
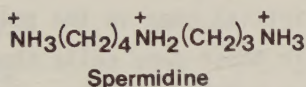
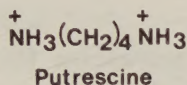


FIGURE 1. Structural formulas of the polyamines. All amino and imino groups are protonated at a physiological pH.

The structure of spermine is particularly well suited to stabilize double-stranded DNA and RNA (4, 5, 9). X-ray diffraction analyses of DNA-spermine fibers have shown that the tetramethylene group bridges across the minor groove of the DNA double helix, while the trimethylene groups bridge the distance between adjacent phosphate groups within one DNA strand (5). In a recent study, Igarashi et al. (10) have shown that spermine binds preferentially to the GC-rich regions in DNA.

Polyamines can also affect the secondary and tertiary structures of the nucleic acids (2, 11). Thus, they have been found to effectively induce the left-handed, double helical conformation of DNA (so-called Z-DNA) occurring in GC-rich regions (12). The conversion between the B-DNA and the Z-DNA conformations appears to be a regulatory mechanism in gene expression. In fact, many of the effects that polyamines exert on various enzymatic reactions *in vitro*, such as those catalyzed by DNA polymerases, RNA polymerases, methylases, nucleotidyl transferases and hydrolases, may be related to conformational changes (11).

2.2 POLYAMINE BIOSYNTHESIS

Prokaryotic and eukaryotic cells all synthesize putrescine and spermidine (2). Nucleated eukaryotic cells in addition synthesize spermine. Spermine is not present in most prokaryotes, but it has been found in two bacterial species, *Pseudomonas aeruginosa* (13) and *Bacillus stearothermophilus* (14). However, in these reports there was no demonstration of actual synthesis of spermine. The recent study by Paulin et al. (15) for the first time demonstrates, that prokaryotes of the genus *Acetobacteria* synthesize spermine. In prokaryotes, the putrescine concentration is at least as high as that of spermidine. In eukaryotes, however, it is usually much lower than those of spermidine and spermine.

Ornithine is the immediate precursor of putrescine (Fig. 2). Nevertheless, the hydrolysis of arginine to ornithine (and urea), catalyzed by arginase, may be considered the initial step in the polyamine biosynthetic pathway (Fig. 2) - at least in those many cells that do not have a complete set of urea cycle enzymes (16). Ornithine is also formed in a reaction catalyzed by transaminidase, in which arginine and glycine are the substrates (Fig. 2). The other product in this transaminidase reaction is guanidinoacetate, which serves as a substrate in the synthesis of creatine, or, conceivably, may be decarboxylated to yield methylguanidine (17). Since methylguanidine is a potent inhibitor of diamine oxidase, it might interfere with the catabolism of putrescine (17) (see section 2.3).

Ornithine is either synthesized within the cell (as described above) or taken up from the blood plasma (18). It acts as a substrate for ornithine decarboxylase (ODC), a key enzyme in polyamine biosynthesis, yielding putrescine and CO_2 (Fig. 2) (18). The ODC activity is very low in nonproliferating mammalian tissues (with the exception of the prostate), but a dramatic increase, which may be several thousandfold, occurs upon growth stimulation (19). The ODC activity in mammalian cells is characterized by a very rapid rate of turnover. Its half-life may be as short as 5-10 min (20, 21).

In certain bacteria and higher plants, there exists an additional pathway for putrescine synthesis: the decarboxylation of arginine to agmatine, catalyzed by arginine decarboxylase, followed by hydrolytic cleavage of agmatine to putrescine and urea, catalyzed by agmatinase

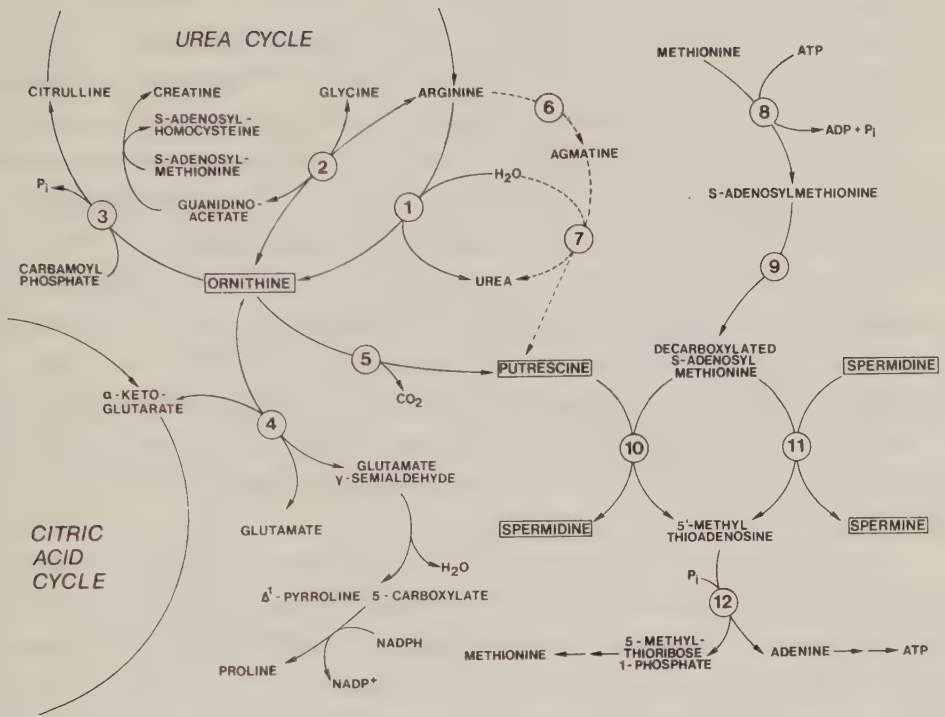


FIGURE 2. Ornithine metabolism and polyamine biosynthesis in mammalian cells (solid lines). The dashed lines represent an alternative pathway for putrescine synthesis in prokaryotic cells.

- 1) arginase (L-arginine amidinohydrolase; EC 3.5.3.1)
- 2) transaminidase (L-arginine-glycine amidinotransferase; EC 2.1.4.1)
- 3) ornithine carbamoyltransferase (carbamoyl phosphate:L-ornithine carbamoyltransferase; EC 2.1.3.3)
- 4) ornithine aminotransferase (L-ornithine:2-oxoacid aminotransferase; EC 2.6.1.13)
- 5) ornithine decarboxylase (L-ornithine carboxy-lyase; EC 4.1.1.17)
- 6) arginine decarboxylase (L-arginine carboxy-lyase; EC 4.1.1.19)
- 7) agmatinase (agmatine amidinohydrolase; EC 3.5.3.11)
- 8) methionine adenosyltransferase (ATP:L-methionine S-adenosyltransferase; EC 2.5.1.6)
- 9) S-adenosylmethionine decarboxylase (S-adenosyl-L-methionine carboxy-lyase; EC 4.1.1.50)
- 10) spermidine synthase (S-methyladenosylhomocysteamine:putrescine aminopropyltransferase; EC 2.5.1.16)
- 11) spermine synthase (S-methyladenosylhomocysteamine:spermidine aminopropyltransferase; EC 2.5.1.)
- 12) 5'-methylthioadenosine phosphorylase (5'-methylthioadenosine:orthophosphate methylthioribosyltransferase)

(Fig. 2) (18). In *Escherichia coli*, both pathways are normally present, although the decarboxylation of ornithine is the major one (22). In animal tissues, however, putrescine is synthesized exclusively from ornithine. Both prokaryotic and eukaryotic ODC, and arginine decarboxylase, require pyridoxal 5'-phosphate for their catalytic activities.

Ornithine is also used as a substrate for at least two additional enzymes. One is the urea cycle enzyme, ornithine carbamoyltransferase, which catalyzes the transfer of a carbamoyl group to ornithine, thus forming citrulline (Fig. 2). The other enzyme, ornithine aminotransferase, catalyzes the first step in the synthesis of proline, the transfer of the ornithine α -amino group to α -ketoglutarate (Fig. 2). The products formed are glutamate and glutamate γ -semialdehyde. In the following step, glutamate γ -semialdehyde spontaneously cyclizes to Δ^1 -pyrroline 5-carboxylate, which is then reduced by NADPH to yield proline.

Methionine, the other amino acid precursor in polyamine biosynthesis, has to be activated by adenylation before it can participate in the synthesis of spermidine and spermine (Fig. 2) (23). This reaction is catalyzed by methionine adenosyltransferase, which employs ATP as the second substrate, yielding S-adenosylmethionine (and inorganic phosphate). The intracellular supply of methionine seems to be rate-limiting in polyamine biosynthesis (11). S-adenosylmethionine both serves as a methyl group donor in about a hundred transmethyating reactions, and as an aminopropyl group donor in the synthesis of spermidine and spermine. The latter reactions are initiated by decarboxylation of S-adenosylmethionine, catalyzed by S-adenosylmethionine decarboxylase (SAMDC), yielding S-adenosyl-5'-deoxy-(5')-3-methylthiopropylamine (decarboxylated S-adenosylmethionine) (Fig. 2) (23). Prokaryotic SAMDC requires Mg^{2+} for its activity, whereas mammalian SAMDC is stimulated by physiological concentrations of putrescine. Upon growth stimulation, the SAMDC activity usually shows the same pattern of changes as does the ODC activity. SAMDC has a half-life that is somewhat longer than that of ODC.

Decarboxylated S-adenosylmethionine seems to be committed for polyamine synthesis. It acts as the immediate aminopropyl group donor in the synthesis of spermidine and spermine, which is catalyzed by spermidine synthase and spermine synthase (Fig. 2) (23). These two

aminopropyl transferases exhibit considerably higher activities than the decarboxylases (ODC and SAMDC) and their half-lives are much longer. Therefore, they are unlikely to regulate the rate of polyamine biosynthesis. Decarboxylated S-adenosylmethionine, on the other hand, appears to have such capacity, because its cellular content is normally rate-limiting. Thus, the cellular decarboxylated S-adenosylmethionine content is usually very low ($< 1 \text{ pmol}/10^6 \text{ cells}$). In cases where the aminopropyl group acceptors (putrescine and spermidine) have been depleted, however, the cellular content of decarboxylated S-adenosylmethionine increases several thousandfold (24-26).

Each of the two synthase reactions produces 5'-methylthioadenosine and one proton in addition to spermidine and spermine (23). 5'-Methylthioadenosine is rapidly cleaved by 5'-methylthioadenosine phosphorylase, yielding 5-methylthioribose 1-phosphate and adenine (Fig. 2) (23). This is the first step in the salvage pathways for methionine and adenine nucleotide synthesis (27). In fact, it represents the only known pathway for *de novo* synthesis of adenine. 5'-Methylthioadenosine, formed in the synthesis of spermidine and spermine, acts as a product inhibitor in these two reactions (27). Spermine synthase is also inhibited by putrescine, which competes with spermidine for the active site of the enzyme (28).

2.3 POLYAMINE INTERCONVERSION AND CATABOLISM

The reactions catalyzed by spermidine synthase and spermine synthase are irreversible, but pathways exist for the conversion of spermine into spermidine and of spermidine into putrescine (25, 29, 30). The mammalian intracellular polyamine oxidase (31) seems to be the catalyst in these interconversion reactions. Before spermidine and spermine can act as substrates for polyamine oxidase, however, they have to be acetylated in a reaction catalyzed by spermidine/spermine N¹-acetyltransferase (25, 29, 32) (Fig. 3). The products formed, N¹-monoacetylspermidine and N¹-monoacetylspermine, are the natural substrates for polyamine oxidase (33). In the polyamine oxidase-catalyzed reactions, 3-acetamidopropanal is formed in addition to putrescine and spermidine, respectively (Fig. 3). Normally, the intracellular activity of spermidine/spermine N¹-acetyltransferase is low, but it is rapidly induced e.g. in the liver following treatment with CCl₄, thioacetamide, growth

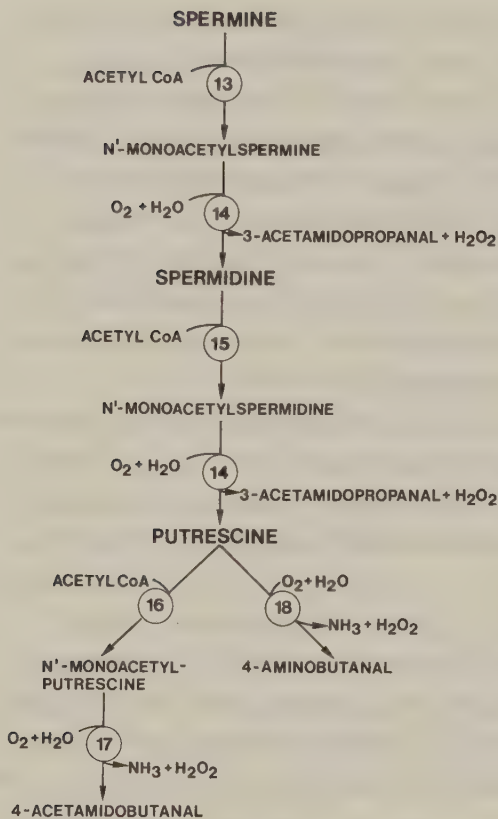


FIGURE 3. Polyamine interconversion and catabolism in mammalian cells. 13) Spermine N¹-acetyltransferase, 14) polyamine oxidase, 15) spermidine N¹-acetyltransferase, 16) putrescine N¹-acetyltransferase, 17) monoamine oxidase (amine:oxygen oxidoreductase; (deaminating) (flavin-containing) EC 1.4.3.4), 18) diamine oxidase (amine:oxygen oxidoreductase; (deaminating) (pyridoxal-containing) EC 1.4.3.6).

hormone or following partial hepatectomy (34, 35). The acetyltransferase has a very short half-life, almost as short as that of ODC, and it appears to be an important regulatory enzyme in the catabolism of spermidine and spermine. The activity and half-life of polyamine oxidase in the cell are comparable to those of spermidine synthase and spermine synthase (36). Therefore, it is not likely to be a rate-controlling enzyme in polyamine catabolism.

N¹-Acetyltransferase adds the acetyl group specifically to the aminopropyl moiety of spermidine, leaving the aminobutyl end of the molecule intact (37). Thus, only N¹-monoacetylspermidine is formed. N⁸-Monoacetylspermidine is formed in reactions, catalyzed by acetyltransferase A and B (38, 39). The spermidine/spermine N¹-acetyltransferase has been purified from the cytosol of CCl₄-intoxicated rat liver (32). The other acetyltransferases are nuclear enzymes and were found in rat and calf liver nuclei (38, 39). The nuclear acetyltrans-

ferases preferentially acetylate histones but also use spermidine and spermine, and to some extent putrescine, as substrates. When spermidine is used as a substrate, the N⁸-monoacetyl derivative is formed. Notably, N⁸-monoacetylspermidine is not a substrate for polyamine oxidase. Instead, it acts as an inhibitor of this enzyme (33).

Putrescine, the end product of the interconversion reactions, can be catabolized further, either through direct oxidative deamination by diamine oxidase or following conversion to N¹-monoacetylputrescine by the nuclear acetyltransferases (Fig. 3). N¹-Monoacetylputrescine is a substrate for mitochondrial monoamine oxidase and is thus oxidatively deaminated (40).

N¹-Monoacetylputrescine, N¹-monoacetylspermidine and N⁸-monoacetylspermidine are all normal tissue constituents (29). The enzymes involved in their formation have been found both in the circulation and intracellularly.

2.4 POLYAMINES AS GROWTH REGULATORS

The first studies, suggesting a role for polyamines in growth, used bacterial systems. Thus, putrescine was found to be an essential growth factor for two strains of bacteria, *Hemophilus parainfluenzae* (41) and *Neisseria perflava* (42) as well as for an ascomycete, *Aspergillus nidulans* (43). Subsequently, a similar dependence on putrescine for growth was observed for several mammalian cell lines, e.g. Chinese hamster kidney cells (44) and human fibroblasts (45, 46).

The activities of the polyamine biosynthetic enzymes, as well as the concentrations of the polyamines, increase precipitously following growth stimulation and remain elevated in rapidly proliferating tissues (47). Usually polyamine synthesis increases prior to DNA synthesis. Whether the polyamines are actually involved in the regulation of DNA synthesis obviously cannot be established on the basis of such circumstantial evidence.

A more direct means of studying the role of the polyamines in cell growth and division, is to deplete the cells of these amines. However, due to the relatively long biological half-lives of spermidine (between 7 and 42 days) and spermine (between 11 and 42 days), it poses a major problem to rapidly deplete cells of these polyamines. Nevertheless, two different approaches are effective in producing a state of

cellular polyamine deficiency. One is to use inhibitors specific for the various enzymes in the polyamine biosynthetic pathway. The other, is to use cell lines that are deficient in these enzymes.

2.4.1 Inhibitors of Polyamine Biosynthetic Enzymes

The development of inhibitors of the polyamine biosynthetic enzymes, has opened a possibility to block polyamine synthesis, thereby decreasing their cellular content (48). Methylglyoxal-bis(guanylhydrazone) (MGBG) was the first polyamine synthesis inhibitor available. It is a potent inhibitor of putrescine-activated SAMDC (49, 50), and is competitive and reversible with respect to the substrate, S-adenosylmethionine. MGBG effectively blocks the synthesis of spermidine and spermine, while the cellular content of putrescine increases. Unfortunately, this drug may not be absolutely specific. At least at higher concentrations, it causes mitochondrial damage and is severely cytotoxic.

The presently most common target of inhibition is ODC, the first and rate-controlling enzyme in the polyamine biosynthetic pathway. Since ODC uses only the L-form of ornithine as substrate, the D-form might be expected to act as an inhibitor of the enzyme. However, it was not a very potent inhibitor (51).

One of the first ODC inhibitors to be synthesized was α -hydrazinoornithine (52, 53). This is a competitive inhibitor of ODC, but may not be entirely specific, because it apparently interferes with other pyridoxal 5'-phosphate-dependent enzymes as well.

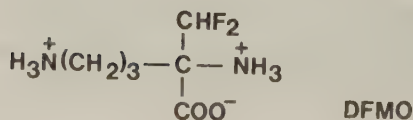
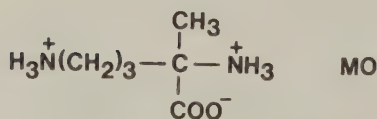
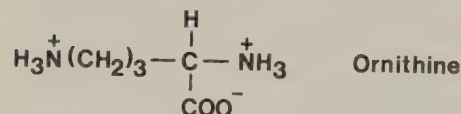


FIGURE 4. Structural formulas of α -methylornithine (MO), a competitive and reversible inhibitor of ODC, and α -difluoromethylornithine (DFMO), an enzyme-activated irreversible inhibitor of ODC. The structural formula of ornithine is shown for comparison.

α -Methylamino acids are potent inhibitors of pyridoxal 5'-phosphate-dependent decarboxylases. Therefore, it was logical to synthesize α -methylornithine (MO) as a possible inhibitor of ODC (Fig. 4). MO proved to be a potent (competitive and reversible) inhibitor of ODC (54). Consequently, MO treatment was found to effectively reduce the cellular concentration of putrescine and spermidine (55-57).

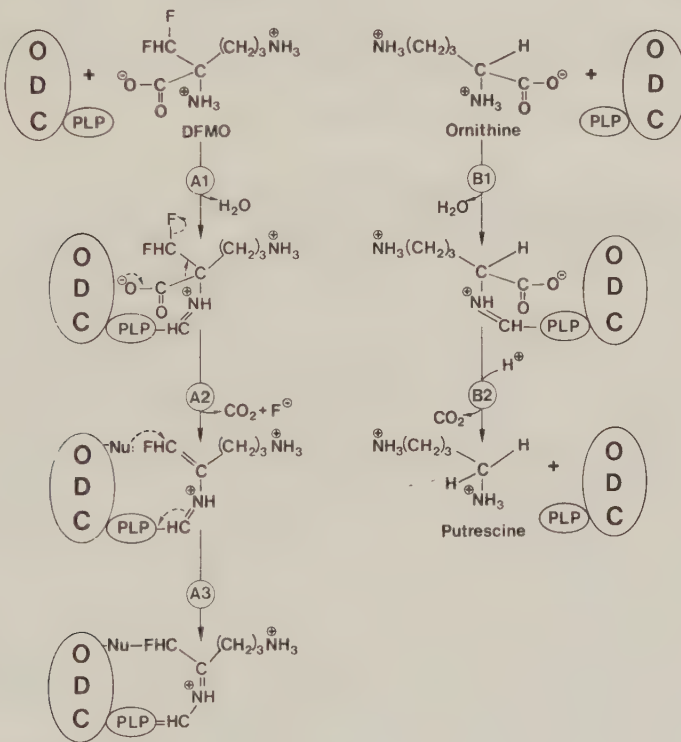


FIGURE 5. Hypothetical mechanism for the enzyme-activated irreversible inhibition of ODC by DFMO (A) compared to the decarboxylation of the normal substrate (B).

The development of α -difluoromethylornithine (DFMO) (Fig. 4) has revolutionized the whole field of polyamine research (58). This irreversible inhibitor was designed according to a new approach, in which a latent reactive group is activated by the target enzyme. The irreversible inactivation of ODC does not occur directly upon binding (58) (Fig. 5).

First, a Schiff's base is formed between the inhibitor and the pyridoxal 5'-phosphate cofactor in the active site of ODC (Step A1, Fig. 5). This reaction is reversible, and basically the same as that occurring when the normal substrate, ornithine, is used (Step B1, Fig. 5). Secondly, DFMO is enzymatically decarboxylated to generate a highly reactive intermediate (Step A2, Fig. 5). Thirdly, this intermediate alkylates a nucleophilic residue at, or near, the active site of ODC, thus covalently binding the inhibitor to the enzyme (Step A3, Fig. 5) (58). When DFMO is decarboxylated (Step A2, Fig. 5), fluoride is also eliminated from the inhibitor. Being a highly specific inhibitor of ODC, DFMO effectively depleted rat hepatoma cells and mouse leukemia cells of their putrescine and spermidine content (59).

2.4.2 Cells Deficient in Polyamine Biosynthetic Enzymes

A different approach for studying the role of polyamines in cell growth and division, is to use mutants with specific defects in the various steps of polyamine biosynthesis. Such mutants have been isolated from *Escherichia coli*. In fact, mutants deficient in arginine decarboxylase (60, 61), agmatinase (60, 62, 63), ODC (62) or SAMDC (64) and mutants deficient in all these enzymes (65) have been developed.

The isolation of eukaryotic cells deficient in the various polyamine biosynthetic enzymes proved to be more difficult. The first eukaryotic polyamine-dependent mutants described were an arginase-deficient (66) and an ODC-deficient (67) mutant of *Neurospora crassa*. From *Saccharomyces cerevisiae*, another primitive eukaryote, mutants deficient in ODC (68, 69), SAMDC (68-70), spermidine synthase (68) or spermine synthase (68) have been isolated.

Recently, the isolation of two polyamine-dependent mammalian cell lines were reported; an arginase-deficient variant (71, 72) and an ODC-deficient mutant (73) of Chinese hamster ovary (CHO) cells.

2.4.3 Cell Cycle Kinetic Effects of Polyamine Deficiency

The cell cycle is the interval between two successive divisions. It may be subdivided into four phases; G₁, S (DNA synthesis), G₂ and M (mitosis) (Fig. 6) (74, 75). During interphase (G₁, S, G₂) essentially all components of the cell are duplicated. These components are then equally divided between the two daughter cells during mitosis. In

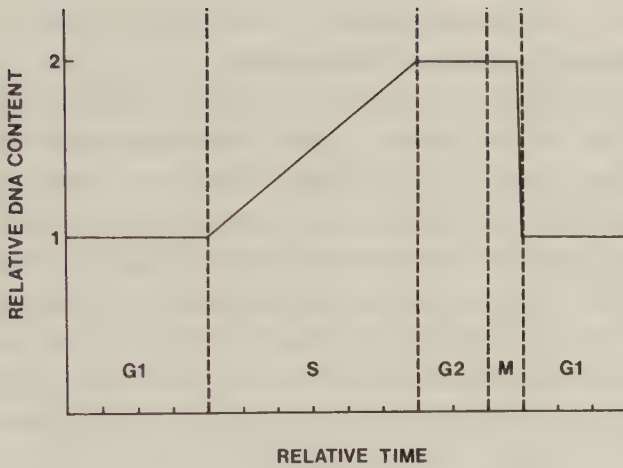


FIGURE 6. Changes in DNA content during the cell cycle. Cells in the G1 phase have a 2C DNA content, cells in the G2 phase a 4C DNA content and cells in the S phase an intermediary DNA content.

addition to actively cycling cells, cell populations usually contain cells that are in a quiescent state (G0 cells).

The first studies on the role of polyamines in cell cycle progression used MGBG and α -hydrazinoornithine as the means of blocking polyamine biosynthesis. These inhibitors exerted a significant antiproliferative effect. As to their effects on cell cycle progression, no consistent pattern was observed among the various experimental systems used (76-87). Nevertheless, a majority of the results indicated that polyamine depletion inhibited DNA synthesis. This effect was attributed to either a decreased frequency of DNA chain initiation (77, 79, 83) or a decreased rate of replication fork movement (76, 78, 81). Since the inhibitors that were used, have been shown to affect other cellular events as well, it cannot be firmly concluded from these studies, that the cell kinetic effects observed are exclusively due to polyamine deficiency.

2.5 INTRACELLULAR DISTRIBUTION OF ODC

The finding that inhibition of polyamine synthesis reduces the rate of DNA synthesis, may be taken to denote that the polyamines have a regulatory function within the cell nucleus. Moreover, inhibition of polyamine synthesis has been found to interfere with ribosomal gene

transcription in polychaete germ cells (88) and with nucleolar formation in developing polychaete embryos (89).

As to the intracellular distribution of the polyamine biosynthetic enzymes, the initial studies (which used fractionated cells and biochemical methods) indicated that ODC and SAMDC were almost exclusively cytosolic enzymes (51, 90-93). Some of these data are compiled in Table 1. In view of the suggested nuclear functions of the polyamines, it seemed probable that shortcomings in the fractionation techniques used, might have confused the issue of ODC localization much the same way as they did the issue of DNA polymerase localization (94). Thus, DNA polymerase activity was initially found almost exclusively in the cytosol.

Table 1. ODC activity in nuclear and cytosolic fractions.

Organ/Tissue	ODC activity		References
	Nuclear fraction	Cytosolic fraction	
	(%) ^a	(%) ^a	
Prostate gland of the rat	4-18	70-94	92
Liver of thioacetamide-treated rat	14	80	51
Liver of partial hepatectomized rat	24	70	51
Liver of normal rat	16	61	91
Liver of growth hormone-treated rat	2	92	91
Mouse fibroblasts	12 ^b	88 ^c	90

^a Based on specific enzyme activities.

^b Karyoplast and ^ccytoplast fractions after cytochalasin B-induced enucleation of cell monolayers.

Recent studies, biochemical as well as autoradiographic, indicate, in contrast to the studies described above, that ODC is present in the nucleus at a significant level, at least in invertebrate germ cells, lower eukaryotes and plants (95-98). A biochemical study on barley seeds even indicates that the ODC activity is localized mainly in the nucleus, tightly bound to chromatin (98). On the basis of these results,

it seemed important to re-evaluate the intracellular localization of the polyamine biosynthetic enzymes in mammalian cells. The isolation of a monospecific ODC antibody (99) and the synthesis of ^3H -DFMO, have made possible a new approach - to study the intracellular localization of ODC by means of microscopic techniques. The antibody binds to ODC protein, including catalytically inactive ODC, thus demonstrating total enzyme protein, whereas ^3H -DFMO binds to the active site of ODC, thus demonstrating only the catalytically active enzyme.

2.6 POLYAMINES AS TUMOR MARKERS

As previously described, the activities of the polyamine biosynthetic enzymes and the concentrations of the polyamines are increased in rapidly proliferating cells, normal as well as tumor cells. Tumor growth is a process that consists of both cell proliferation and cell death. As a consequence of cell death, intracellular components, including polyamines, should be expected to be released into the circulation. In fact, an increased urinary excretion of polyamines was observed in patients with advanced cancer (100, 101). It was proposed that this increase was a result of leakage of putrescine from proliferating cells and spermidine from dead cells (102). At variance with this hypothesis, results derived from experimental tumors suggested that increased putrescine and spermidine levels in extracellular fluids were due to release from dead or dying tumor cells only (103, 104).

As for most other potential cancer markers, it was found that serum and urine polyamines increased even during various nontumor conditions, characterized by both cell proliferation and cell death. Their levels were found to vary during the menstrual cycle (105) and to increase during normal pregnancy (106, 107), as well as in patients with diseases other than cancer, such as psoriasis, cystic fibrosis, liver insufficiency, rheumatoid arthritis and hemolytic anemia (108-111). It was thus apparent that the analysis for polyamines in physiological fluids was not applicable in general cancer screening.

Nevertheless, the analysis of polyamines may serve as a diagnostic aid in combination with established techniques in cases of suspected cancer. Polyamine analysis may also be used in the evaluation of therapeutic effectiveness, at least for certain types of cancer. Studies by Marton et al. (112-114) indicate that cerebrospinal fluid polyamines

have a prognostic value in patients with medulloblastoma. Their results show an excellent correlation between polyamine concentration in cerebrospinal fluid and the clinical status of the patient.

Polyamine levels in physiological fluids decrease following surgical removal of a tumor (Fig. 7). After successful chemotherapy, polyamine levels increase initially and then decrease towards a normal level. When patients do not respond to chemotherapy, the physiological fluid polyamines remain at their elevated levels. The analysis of polyamines in cancer patients may also reveal a relapse of a tumor (115). In fact, the increase in polyamine levels may precede clinical evidence of regrowth of a tumor by as much as 40 weeks (113).

Although clinical studies have established a relationship between extracellular polyamines and tumor growth, an experimental approach is required to determine whether increased polyamine excretion is causally related to tumor cell growth and/or tumor cell death.

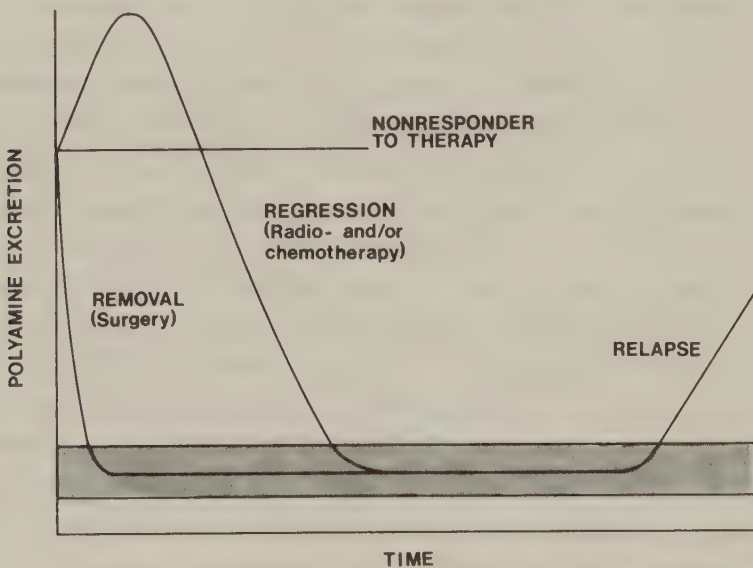


FIGURE 7. Generalized pattern of changes in polyamine excretion in cancer patients following surgical removal of the tumor, following treatment with radio- and/or chemotherapy (responder vs. nonresponder), and during regrowth of the tumor. The normal variation in polyamine excretion is indicated by the dotted area.

3. THE PRESENT STUDY

3.1 SPECIFIC AIMS

Experiments, in which the first developed polyamine synthesis inhibitors (MGBG and α -hydrazinoornithine) were used, indicate that the polyamines are necessary for cell growth and division. The possibility remains, however, that the effects of these inhibitors are partly unrelated to polyamine deficiency.

A major aim of the present study was therefore to use the new and highly specific polyamine synthesis inhibitor, DFMO, as well as a polyamine-deficient (arginase-deficient) mammalian cell line, in order to analyze more specifically the role of the polyamines in cell growth (I; II); particularly in the traverse of the cell cycle (II).

Recent observations, made in our laboratory and in those of others, indicate that the polyamines and/or their biosynthetic enzymes have a regulatory function within the cell nucleus. Therefore, it seemed surprising that ODC should be a cytosolic enzyme, as suggested by biochemical fractionation analyses.

A major aim of the present study was to re-evaluate the question of ODC localization, using two novel approaches - (indirect) immunofluorescence localization of a monospecific ODC antibody and autoradiographic localization of bound ^3H -DFMO (III).

Based on the contention that polyamine excretion is positively correlated with tumor burden, polyamine analyses have been introduced in the clinic in order to monitor tumor growth, regression and relapse.

A major aim of the present study was to analyze more specifically (with the use of animal models that can be experimentally manipulated) the cause(s) of increased urinary polyamine excretion observed in association with tumor growth (IV; V) with special emphasis on the possible relation to cell proliferation and cell death (VI).

3.2 POLYAMINES AS GROWTH REGULATORS - EXPERIMENTAL SYSTEMS

To study the role of polyamines in cell growth and cell cycle kinetics, cells were depleted of their polyamines using two different approaches: irreversible inhibition of ODC with DFMO (I), and ornithine and polyamine starvation of an arginase-deficient cell variant (II). In the study of the intracellular localization of ODC, an ODC overproducing Chinese hamster ovary (CHO) cell mutant was used (III). The various cell lines were grown in specific media (see below) and were incubated at 37°C in a high-humidity atmosphere of 5% CO₂ in air.

3.2.1 Ehrlich Ascites Tumor Cells (I)

The Ehrlich ascites tumor originates from a solid transplantable mammary carcinoma of the mouse (116). It was converted into an ascites tumor (117) and distributed to laboratories all over the world by Dr. Hans Lettré. Cells of a hyperdiploid subline of the Ehrlich ascites tumor (Ehrlich-Lettré-Diploid, Copenhagen) (ELD) were routinely transplanted intraperitoneally every 7th day to inbred male NMRI mice. This ELD strain was obtained from the Department of Genetics, University of Lund in 1969 (118) and has been maintained since then by serial transplantations.

For the establishment of cultures, ELD cells were withdrawn from a mouse six days after tumor transplantation and adapted to suspension culture growth (without agitation). Swim's S-77 growth medium containing 10% fetal calf serum and the various supplements described in paper I was used. ELD cells grew in this medium for about 30 passages without changing their characteristics. To study the role of polyamines in cell proliferation, a 5 mM concentration of either MO or DFMO was used to deplete the ELD cells of their polyamines (I).

The ELD cells were found to grow equally well in the presence of 0.2% bovine serum albumin instead of fetal calf serum. Therefore, ELD cells are now maintained in Ham's nutrient mixture F12 and Eagle's minimum essential medium (MEM) (1:1 by volume) supplemented with 0.2% bovine serum albumin, penicillin (50 IU/ml)-streptomycin (50 µg/ml) mixture and 20 mM NaHCO₃. ELD cells have been grown in this medium for more than 200 passages.

3.2.2 Arginase-Deficient CHO Cells (II)

The polyamine-dependent (arginase-deficient) variant (CHO-A7) used was isolated from wild-type CHO cells by Dr. Pirkko Pohjanpelto (71, 72). Wild-type CHO cells (obtained from American Type Culture Collection) were routinely cultured in the presence of 10% fetal calf serum and 0.5 μ M putrescine. They were adapted to grow without serum, in a 1:1 (v/v) mixture of Ham's F12 medium and Eagle's MEM supplemented with 0.1% bovine serum albumin (giving a final putrescine concentration of 0.5 μ M) by gradually decreasing the serum concentration. Finally a cell variant was obtained, which grew well without serum but was dependent on ornithine and polyamines for growth (71). This dependence on ornithine and polyamine supplementation was found to be due to arginase deficiency (72). Notably, the parental cells are also arginase-deficient, but obtain ornithine due to the presence of arginase in the serum.

The CHO-A7 cells are routinely cultivated in gelatinized petri dishes in a 1:1 (v/v) mixture of Ham's F12 medium and Eagle's MEM supplemented with 0.2% bovine serum albumin, 0.1 mM ornithine, penicillin (50 IU/ml)-streptomycin (50 μ g/ml) mixture and 20 mM NaHCO_3 (giving a final putrescine concentration of 0.5 μ M). To obtain a polyamine-deficient state, CHO-A7 cells were grown in a medium that had not been supplemented with putrescine or ornithine (II). To inhibit the superinduction of ODC, that occurs in CHO-A7 cells during polyamine starvation, the cells were simultaneously treated with 5 mM DFMO.

3.2.3 ODC Overproducing CHO Cells (III)

The ODC overproducing mutant used (C51.19) was isolated following mutagenesis of wild-type CHO cells by Choi and Scheffler (119). Several million wild-type cells (CHO-Toronto) were mutagenized with ethylmethane sulfonate (400 μ g/ml, 24 hr) and after a few days of growth in the absence of the drug, 8.5 mM MO was added to the medium. The small number of resistant clones, that grew up at this concentration of MO, were isolated and maintained in a medium containing 10 mM MO. One of these clones (C51) was subjected to further selection in the presence of 0.85 mM DFMO. Several resistant clones were isolated, one of them being C51.19. These mutant cells grow well in a medium supplemented with 10 mM MO or 0.42 mM DFMO, with the phenotype of the cells being stable even during nonselective conditions. Resistance

to MO and DFMO was found to be associated with overproduction of ODC (119). The mutant cells exhibited about 10-fold higher levels of induced ODC as compared to the parental CHO cells. The enzyme itself is apparently unaltered by the mutation (119).

The mutant cells were routinely cultured in Dulbecco's modified Eagle's MEM supplemented with 10% fetal calf serum, 1 mM proline, penicillin (50 IU/ml)-streptomycin (50 μ g/ml) mixture and 44 mM NaHCO_3 . These mutant cells do not form a regular monolayer when growing to a high density. In fact they pile up on each other already in relatively sparse cultures. For the experiments, the cells were grown in high-serum (10%) medium containing 10 mM MO for 48 hr (III). Then the cell-layer was washed with serum-free medium and incubated in low-serum (0.1%) medium. After 16 hr, the medium was changed to high-serum (10%) medium without MO. Five hours later maximum ODC activity was observed.

3.3 POLYAMINES AS TUMOR MARKERS - EXPERIMENTAL SYSTEMS

To analyze the cause(s) of the increased urinary polyamine levels observed in association with tumor growth, various animal models were used. Effects of tumor burden and tumor regression were studied using a transplantable adenocarcinoma (IV) and a hormone-dependent mammary carcinoma (V). The effects of tumor vascularization were studied following intrahepatic and s.c. inoculation of the adenocarcinoma (IV). Finally, a nontumor model was used to assess whether polyamine excretion changes as a function of cell death or cell proliferation (VI).

3.3.1 Transplantable Adenocarcinoma (IV)

The tumor used was a transplantable rat adenocarcinoma (NG-W1) (IV). It originated in the colon of Wistar rats following N-methyl-N'-nitro-N-nitrosoguanidine treatment. To maintain the tumor, it was transplanted intrarenally into Wistar rats every 10 days as a coarse cell suspension.

For the experiments, a monodisperse cell suspension of the tumor was prepared as follows: the tumor was removed from the kidney, cut into small pieces and trypsinized. The trypsinization was stopped by the addition of rat serum. The percentage of viable cells was determined by the trypan blue exclusion method. The cell suspension was then diluted to yield 1.0×10^6 viable tumor cells per 0.1 ml inoculum.

To study whether the urinary polyamine excretion would be

dependent on the vascularization of the tumor, the NG-W1 tumor was transplanted either intrahepatically or s.c. into male Wistar rats (IV). This experiment is based on the fact that intrahepatic tumors have a much better vascularization than do s.c. tumors (120).

One group of rats was inoculated intrahepatically. Thus, the abdominal cavity was opened through a midline incision and the cell suspension (1.0×10^6 viable tumor cells) was inoculated just below the capsule of the central lobe of the liver. Another group of rats received an s.c. inoculation of the same tumor cell suspension (1.0×10^6 viable tumor cells) in the back.

At various times after inoculation, tumor size was measured using vernier calipers. Intrahepatic tumors were measured during laparotomy. Two perpendicular diameters were used to estimate the tumor volume according to a formula suggested by Steel (121):

$$V = \frac{\pi(\text{mean diameter})^3}{6}$$

The NG-W1 tumor has not been observed to metastasize. Therefore, the above estimate of tumor volume should be a close approximation of the total tumor burden. As to the s.c. tumors, the skin thickness gives a certain contribution to diameter measurements, thereby causing an overestimation of tumor volume. Therefore, a constant of 1.5 mm, roughly corresponding to double skin thickness, was subtracted from each diameter measurement before calculating the tumor volume (121). The resulting growth curve should be a close approximation of the actual growth of the s.c. tumor.

3.3.2 Hormone-Dependent Mammary Carcinoma(s) (V)

7,12-Dimethylbenz(a)anthracene (DMBA)-induced rat mammary carcinomas exhibit many biochemical and histological characteristics, that are similar to those of human mammary carcinomas (122, 123).

DMBA induces mammary carcinomas when administered as a single intragastric dose (20 mg of DMBA in 1 ml of sesame oil) into virgin (50 days old) Sprague-Dawley rats (V; 124). The mammary carcinomas arise from the incompletely differentiated terminal ductal structures (terminal end buds), which are present in the developing mammary gland of young virgin rats (123). When the rat is between 30 and 55 days old, it exhibits the highest terminal end bud DNA-labeling index (123).

This coincides with the age at which DMBA administration causes the highest incidence of mammary carcinomas (125).

Within 45 days after DMBA administration mammary tumors were palpable in almost all rats (V). The tumor size was estimated from then on, once or twice weekly, using vernier calipers at two perpendicular diameters. A constant of 1.5 mm, roughly corresponding to double skin thickness, was subtracted from each diameter measurement before calculating the tumor volume (121). The volume of each tumor was calculated according to a formula suggested by Steel (121):

$$V = \frac{(\text{length} \times \text{width}^2)}{2}$$

The DMBA-induced carcinomas are dependent on estrogen, prolactin and other hormones for growth (122). Since up to 90% of the established mammary carcinomas are estrogen-dependent, it is possible to induce regression by subjecting the rats to bilateral ovariectomy.

3.3.3 CCl₄ Induction of Liver Cell Death and Proliferation (VI)

CCl₄ intoxication of the rat is a useful non-tumor experimental system for studying events associated with both cell necrosis and proliferation. Following a sublethal dose of CCl₄, there is extensive necrosis in the liver, but no other organs seem to be damaged (126, 127). Liver necrosis is gradual; the cells in the central zone are subjected to a lethal dose of CCl₄ and die, the cells in the midzone may recover due to a sublethal dose and the cells in the periportal area recover rapidly (128, 129). The cells in the periportal area are responsible for the subsequent liver regeneration. From 24 hr on, the cells are active in the regeneration process, thus repopulating the liver.

Female Wistar rats were injected intraperitoneally with a single dose of CCl₄ dissolved in olive oil (0.5 ml of CCl₄/kg body wt) (VI). Following administration, CCl₄ is rapidly metabolized by the liver to several reactive products, which seem to be the primary toxins (126, 129).

3.4 ANALYTICAL PROCEDURES

3.4.1 Collection of Cells

Cell numbers were counted in a Coulter Counter (I) or in a

hemocytometer (II; III) and samples were removed for polyamine analysis (Section 3.4.7), enzyme assays (Section 3.4.4) (I-III) and flow cytometric analysis (Section 3.4.2 and 3.4.3) (II). The cells were harvested by centrifugation. For polyamine analysis, cells were stored at -20°C and for enzyme assays at -70°C . For flow cytometric analysis, cells were immediately fixed as described in Section 3.4.2.

3.4.2 Cell Preparative Procedure for Flow Cytometric Analysis

Single-cell suspensions ($1-2 \times 10^6$ cells) were fixed in 1 ml of absolute ethanol (-20°) and stored at 4°C until analysis (less than a month). After pelleting, the cells were resuspended in 1 ml of 0.1% RNase (75 U/ml) in Buffer A (0.1 M Tris-HCl buffer containing 70 mM NaCl and 5 mM Na_2EDTA , adjusted to pH 7.5 and filtered through a $0.45\ \mu\text{m}$ Millipore filter) and kept at room temperature for 5 min. After pelleting, the cells were resuspended in 1 ml of 0.5% pepsin (5 E/ml) in 55 mM HCl and incubated for 10 min at 37°C . The resulting cell nuclei were cooled on ice (5 min), pelleted and resuspended in 1 ml of Buffer A and passed through a nylon mesh with a pore size of $50\ \mu\text{m}$, using a 1 ml syringe. For quantitative fluorescent staining, pelleted cell nuclei were finally resuspended in 1 ml of 0.005% (75 μM) propidium iodide in Buffer A supplemented with 0.6% Nonidet P 40 (NP 40). The stained cell nuclei were finally passed through a $50\ \mu\text{m}$ nylon mesh.

Between each step in the protocol described, the cells were centrifuged at 250 xg for 5 min and the supernatant decanted. Unless specifically stated, all procedures were performed at $0-4^{\circ}\text{C}$.

Propidium iodide, an analog of ethidium bromide, selectively and quantitatively intercalates into the double-stranded regions of DNA and RNA (130). It can be used as a quantitative stain for cellular DNA after treatment of ethanol-fixed cells with RNase (131). The addition of RNase and NaCl (at concentrations of 10 mM or greater) eliminates the binding of the fluorochrome to RNA and to secondary binding sites in DNA. At low ionic strength, the electrostatic interaction of the two positive charges of propidium iodide and the negative charges of the DNA phosphates increase.

EDTA and pepsin was added to optimize the accessibility of DNA to the fluorochrome. A substantial increase in dye binding to DNA occurs if cells (unfixed or fixed) are pretreated with pepsin prior to

staining (132). Pepsin treatment also eliminates cytoplasmic fluorescence. The nonionic detergent (NP 40) was added to the staining solution to remove residual cytoplasm that might cause clumping and non-specific staining (133).

3.4.3 Flow Cytometric Analysis

Propidium iodide-stained nuclei, at a density of about 1×10^6 per ml, were analyzed on a Cytofluorograph System 50-H (Ortho Instruments) equipped with a 4 W argon-ion laser (Model 95). The laser line at 488 nm (450 mW power output) was used for excitation. The excitation maximum for propidium iodide shifts from about 490 to 540 nm and the emission maximum from about 635 to 625 nm (orange-red fluorescence) upon binding to cellular DNA (134). Fluorescence emission was detected between 625 nm and infrared. About 2×10^5 nuclei from each preparation were analyzed. The impulses were sorted by a 512 multichannel analyzer and processed with a PDP 11/34 computer. Nuclear doublets were excluded by electronic threshold settings.

In the DNA histograms obtained, the peak at approximately twice the modal channel number of G1 cell nuclei represents G2 cell nuclei. Obviously, mitotic cell nuclei should appear in this second peak. However, since most mitotic cells lack a nuclear membrane, they are likely to be destroyed by pepsin (132). The nuclei of late-telophase cells may separate and appear in the first peak of the histogram. The percentages of cells in each phase of the cell cycle were derived from the DNA distributions essentially as described by Dean (135).

3.4.4 ODC and SAMDC Assays

The activities of ODC and SAMDC were followed by measuring the release of $^{14}\text{CO}_2$ from DL(1- ^{14}C)ornithine and S-adenosyl-L(carboxyl- ^{14}C)-methionine, respectively. The cells were disrupted by sonication in 10 mM Tris-HCl buffer (pH 7.2) containing 5 mM dithiothreitol, 0.5 mM Na_2EDTA and 0.05 mM pyridoxal 5'-phosphate. The ODC activity was assayed for in the presence of a saturating (1 mM) level of ornithine and the SAMDC activity in the presence of a nonsaturating S-adenosyl-methionine concentration (11 μM) and a saturating (2.5 mM) level of putrescine, the enzyme activator (136).

3.4.5 ODC Localization by Immunofluorescence

The ODC overproducing CHO cells were grown on glass coverslips according to the experimental protocol described in Section 3.2.3. Five hours after serum stimulation, the cells were fixed in 4% para-formaldehyde in Tyrode's balanced salt solution (pH 7.2) for 1 hr at 4°C. The fixative was supplemented with 5 mM dithiothreitol, since it stabilizes ODC (137) and appears to enhance the fluorescence (138). After fixation, the cells were rinsed thoroughly in Tyrode's balanced salt solution containing 10% sucrose (pH 7.2, 4°C) and then stored at -20°C.

The cells were processed for indirect immunofluorescence mainly as described by Coons et al. (139). To make the cells permeable to the antibodies, all solutions were prepared in permeabilizing buffer; 0.25% Triton X-100 and 0.25% human serum albumin in phosphate-buffered saline (PBS). The fixed cells were incubated with monospecific ODC antiserum (99), diluted 1:640, for 3 hr at room temperature, and then rinsed in PBS. The site of the antigen-antibody reaction in the cell was visualized by incubation for 30 min with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG, diluted 1:20. Finally, the cells were rinsed and mounted in PBS-glycerol (1:1). Specificity controls were run in parallel according to Sternberger (140). They consisted of comparison of fluorescence of cells exposed to (A) inactivated (incubated with homogeneous mouse kidney ODC; 100 µg/ml of diluted antiserum) ODC antiserum and (B) ODC-unrelated (anti-gastrin) antiserum. Cells were also incubated with FITC-conjugated goat anti-rabbit IgG only (without a primary antibody) and examined for positive fluorescence.

3.4.6 ODC Localization by Autoradiography

The ODC overproducing CHO cells were grown on glass slide pieces or directly on the plastic substrate according to the experimental protocol described in Section 3.2.3. One hour after serum stimulation, ³H-DFMO was added to the growth medium and the cells were grown in the presence of the labeled inhibitor for 4 hr. Following labeling, the cells were subjected to a chase for 0, 15, 30 or 60 min in growth medium supplemented with 10 mM DFMO (unlabeled). The cells were fixed in 2% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.3) for 1 hr at 4°C.

The cells were then post-fixed in 1% OsO_4 in 0.2 M cacodylate buffer (pH 7.3) for 1 hr at 4°C . Following rinsing in buffer, the cells were dehydrated and stained in ethanol (90%) containing 1% phosphotungstic acid and 0.5% uranyl acetate. Then the cells were embedded in Epon 812, detached from the plastic/glass substrate and sectioned for light ($1\ \mu\text{m}$ sections) and electron (ultrathin sections) microscope autoradiography. Cells that were fixed directly on plastic substrate were rinsed in 90, 95 and 97% 2-hydroxypropyl methacrylate (HPMA) in water and then with a 1:1 mixture of HPMA and Epon 812 before the final embedding in Epon 812.

The $1\ \mu\text{m}$ sections were coated with Ilford K2 liquid nuclear emulsion, according to the dipping method. The autoradiographs were exposed for 2-3 weeks at 4°C , developed in Kodak D 19 (5 min, 18°C), briefly rinsed in distilled water and fixed in Kodak F 24 (6 min, 18°C). They were finally stained in a solution containing Richardson's azure II and methylene blue.

The ultrathin sections were first coated with a protective carbon layer and then with Ilford L4 liquid nuclear emulsion, according to the loop method. The autoradiographs were exposed for 1-3 months at 4°C , developed in Kodak D 19 (2 min, 20°C), rinsed in distilled water (30 sec), fixed in newly made 15% $\text{Na}_2\text{S}_2\text{O}_3$ (3 min, 20°C) and finally rinsed in distilled water.

3.4.7 Polyamine Analysis

Cellular polyamines were analyzed in homogenates obtained by sonication either in 0.2-0.4 M perchloric acid (PCA) (II; III), or in 10 mM Tris-HCl buffer (pH 7.2) containing 5 mM dithiothreitol, 0.5 mM Na_2EDTA and 0.05 mM pyridoxal 5'-phosphate (I). An aliquot of the latter homogenate was mixed with an equal volume of 0.4 M PCA. All PCA extracts (I-III) were kept on ice for 1 hr and were then centrifuged at $1000\ \text{xg}$ for 10 min.

The polyamines were analyzed quantitatively using thin-layer chromatographic methods. They were allowed to react with 1-dimethyl-amino-naphthalene-5-sulfonylchloride (DANS-Cl), according to the following protocol. A $200\text{-}\mu\text{l}$ -aliquot of each sample was mixed with $400\ \mu\text{l}$ of a DANS-Cl solution (30 mg/ml of acetone) and $100\ \mu\text{l}$ of a saturated Na_2CO_3 solution. The Na_2CO_3 is added to the reaction mixture, since DANS-Cl reacts more favorably with amino groups during alkaline

conditions (around pH 10) (141, 142). DANS-Cl reacts rapidly with primary and secondary amino groups, but the reaction mixture was kept for 16 hr in the dark, at room temperature and in the presence of an excess of the reagent, to ascertain a quantitative dansylation of the polyamines. Since DANS-Cl is easily hydrolyzed by silica gel, thus causing bluegreen fluorescent streaks on the chromatograms (142), excess DANS-Cl was removed by reaction with proline (15 mg in 100 μ l). After 30 min in the dark, the dansylated polyamines were extracted into 500 μ l of toluene by vigorous shaking and the layers were separated by centrifugation. A 5- μ l-aliquot of each toluene extract was applied on a pre-activated (1 hr at 110°C) Silica Gel 60 thin-layer chromatographic plate, using a Hamilton micro-syringe pipet. Mixtures of the three polyamines were used as standards. Three different concentrations were dansylated in parallel with the samples and were included on each plate to give a final amount of 50, 100 and 200 pmoles.

The thin-layer chromatographic plates were developed in ethylacetate/cyclohexane (2/3, v/v) in the dark for about 90 min and then dried for 30 min in a desiccator in the dark (II; III). Since pyridoxal 5'-phosphate (included in the homogenization medium used in paper I) interferes with the polyamines when eluted in ethylacetate/cyclohexane, a different elution medium (chloroform/isopropanol; 25/1, v/v) was used in this particular study.

The dansylated polyamines were analyzed using an Aminco-Bowman spectrophotofluorometer. The excitation maximum was at 340 nm and the emission maximum at 505 nm for the dansylated polyamines. The spectrophotofluorometer was equipped with a Thin-Film Chromatograph Scanner and an X-Y-recorder.

Urinary polyamines were analyzed in one-ml aliquots of 24-hr urine collections (described in Section 3.4.8). After hydrolysis with an equal volume of 12 M HCl at 110°C for 14-16 hr (IV; VI), the samples were neutralized with solid Na₂CO₃ and centrifuged at 2000 xg for 10 min. A 200- μ l-aliquot of each supernatant was dansylated as described above (106). The thin-layer chromatography plates were developed in chloroform/triethylamine (11/1, v/v) in the dark for about 60 min and then dried for 30 min in a desiccator before analysis. In one study (V), the 24-hr urines were hydrolyzed for 6 hr only. This hydrolysis time was sufficient and gave the same result as the longer (16 hr) one.

3.4.8 Collection of 24-hr Urines

For collection of 24-hr urines, the rats were maintained in Econo Model E-1100 metabolism units (Maryland Plastics, New York) modified to minimize evaporation. To prevent bacterial growth, 6 M HCl (0.5 ml) was added to each flask before collection was started. After measuring the total urine volume, aliquots were centrifuged at 1000 xg for 10 min. The supernatants were then stored at -20°C until analysis of polyamines (Section 3.4.7) and creatinine (Section 3.4.9).

3.4.9 Creatinine Analysis

Urinary creatinine concentrations were determined colorimetrically according to the Jaffé reaction, using a GEMSAEC Centrifugal Analyzer (Electro-Nucleonics, Inc.). Creatinine is reacted with an alkaline picric acid solution to form an amber-colored compound, with an absorbance maximum at 520 nm.

3.5 POLYAMINES AS GROWTH REGULATORS - RESULTS AND DISCUSSION

3.5.1 Effects of Polyamine Synthesis Inhibitors and Polyamine Starvation on Intracellular Polyamine Biosynthesis and Content

Treatment with DFMO caused a marked inhibition of the ODC activity both in ELD cells (I) and in CHO-A7 cells (II). Even though DFMO is an irreversible inhibitor of ODC, no complete inhibition of the enzyme activity was achieved in ELD cells. Nevertheless, in CHO-A7 cells (II), as well as in rat hepatoma cells (56, 59), the increase in ODC activity occurring upon growth stimulation was almost completely prevented by the addition of DFMO. The presence of a significant ODC activity in DFMO-treated ELD cells may be due to their high rate of turnover of ODC (about 20 min) or an increased rate of transcription.

Rather than inhibiting the ODC activity in ELD cells, MO treatment caused a marked elevation, as determined *in vitro* (I). This increase in ODC activity is probably a result of stabilization of the enzyme by the reversible inhibitor (143). The enzyme might be less susceptible to proteolytic digestion when bound to MO. In the *in vitro* ODC assay, the inhibitor is diluted as much as 700-fold, which implies that its concentration in the reaction mixture amounts to 8 μ M at the most. The dilution appears to relieve the inhibition of ODC activity

exerted by MO in the cell and liberate the accumulated amount of enzyme.

Ornithine and polyamine starvation of CHO-A7 cells caused a marked increase in the SAMDC activity compared to the control (II). This elevation was observed even in the presence of DFMO. In fact, DFMO treatment alone also causes an increase in SAMDC activity (24). The increase in SAMDC activity is probably a result of stabilization of the enzyme and may be a consequence of spermidine depletion (144, 145). Thus, spermidine appears to be a negative effector of SAMDC in the cell. Putrescine, on the other hand, is an activator of the enzyme (146).

By inhibiting the ODC activity, DFMO caused a rapid depletion of putrescine and spermidine in ELD cells (I) and in CHO-A7 cells (II). When CHO-A7 cells were starved for ornithine and polyamines (without addition of DFMO), they were also rapidly depleted of putrescine and spermidine (II). MO treatment of ELD cells only caused an initial decrease in putrescine content (I). Even though the *in vitro* assay did not reveal any inhibition of ODC activity, the decrease in putrescine content suggests that it was in fact inhibited to some extent in the cells. The spermidine content of the ELD cells was not markedly affected by MO treatment (I). This is at variance with results from other cells, in which the putrescine as well as the spermidine content was reduced upon MO treatment (56, 147).

The cellular spermine content was significantly increased in both MO- and DFMO-treated ELD cells (I), as well as in polyamine-starved (with or without DFMO) CHO-A7 cells (II). This may be due to the fact that putrescine inhibits spermine synthase at physiological concentrations by competing with spermidine (28). Due to the decreased putrescine content (following DFMO treatment and polyamine starvation), spermidine may compete more favorably for the active site of spermine synthase. Thus, spermine may be formed at an increased rate until the spermidine content becomes limiting. The increase in spermine content was much more pronounced in MO-treated than in DFMO-treated ELD cells, probably because spermidine was continuously present at a normal concentration (I).

3.5.2 Compensatory Mechanisms in Polyamine-Starved CHO-A7 Cells

Not only SAMDC but also ODC was found to be superinduced in CHO-A7 cells growing in ornithine- and polyamine-free medium (II). In

the presence of a high ODC activity and in the absence of ornithine, structurally related substrates may be used. Thus, ODC has been found to catalyze the decarboxylation of lysine, yielding cadaverine (148, 149). Since cadaverine can act as an aminopropyl group acceptor in the spermidine synthase-catalyzed reaction, aminopropylcadaverine may be formed in a subsequent step (150). Similarly, bisaminopropylcadaverine may be formed in the spermine synthase-catalyzed reaction (150). In fact, cadaverine and aminopropylcadaverine has been shown to appear during the course of ornithine and polyamine starvation of CHO-A7 cells (151). To prevent the formation of cadaverine and its derivatives, the catalytic activity of ODC was inhibited by the addition of DFMO at the onset of starvation (II).

3.5.3 Effects of Intracellular Polyamine Depletion on Cell Growth

Polyamine depletion of ELD and CHO-A7 cells caused a continuous decrease in growth rate (I; II). This effect on growth was not obvious until 24 hr after seeding, i.e. when the cells had traversed almost two cell cycles (I; II). If a 1, 2, 3 or 6 hr lag was introduced between growth stimulation and DFMO treatment, the inhibition of ELD cell growth was gradually decreased (Anehus, Oredsson & Heby, unpubl.). When DFMO was added 24 hr after seeding, i.e. after maximal accumulation of polyamines, there was no inhibition of ELD cell growth (I). This indicates that when the cells have expanded their polyamine pools, they are resistant to the action of the inhibitor.

Omission of ornithine and polyamines from the growth medium, caused a significant inhibition of CHO-A7 cell growth (II). Due to superinduction of ODC, these cells synthesize cadaverine and aminopropylcadaverine, which may be able to substitute for putrescine and spermidine. Although cadaverine is less effective in stimulating growth than are putrescine, spermidine and spermine (56, 151, 152), it may at least partly, counteract growth arrest. Accordingly, prevention of ODC superinduction by treatment with DFMO, potentiated the inhibition of CHO-A7 cell growth (II).

When ELD cells were treated with MO, their growth was not affected (I). This is at variance with results from other cell lines, which were significantly inhibited in their growth upon treatment with 5 mM MO (55, 147), i.e. the same concentration that was used in the present study (I). That MO did not inhibit proliferation of ELD cells (I) may

be explained by its failure to decrease the cellular spermidine content and the comparatively much higher putrescine and spermidine content of these cells.

The present study (I; II) clearly shows, that cells are dependent on polyamines (at least putrescine and spermidine) for the maintenance of optimal growth and division. This conclusion has been corroborated by many subsequent studies, in which various *in vivo* and *in vitro* systems were used (56, 59, 153-156).

That growth inhibition, induced by polyamine synthesis inhibitors or polyamine starvation, is indeed a result of polyamine depletion is indicated by experiments, in which growth inhibition was completely reversed by the addition of putrescine, spermidine or spermine (56, 59, 71, 153-155, 157-159). Growth inhibition may be partly reversed by the addition of some putrescine and spermidine homologs (higher and lower) (56, 150, 159). However, diaminopropane (a lower homolog of putrescine) was unable to reverse the DFM0-induced inhibition of ELD cell growth (157).

3.5.4 Effects of Intracellular Polyamine Depletion on Cell Cycle Progression

When CHO-A7 cells were starved for ornithine and polyamines, they accumulated in the S and G2 phases of the cell cycle (II). Simultaneous treatment with DFM0 caused a somewhat more pronounced accumulation of cells in these cell cycle phases. If the duration of ornithine and polyamine starvation was extended (10 days) and polyamine synthesis simultaneously suppressed by DFM0, there was a further shift toward a G2 phase DNA content (II) (Fig. 8, Table 2). It has to be taken into account, however, that some of the cells with a 4C DNA content may be tetraploid cells in the G1 phase of the tetraploid cell cycle. In fact, large cell nuclei have been found to occur during ornithine and polyamine starvation of CHO-A7 cells (160).

Some experiments have suggested, that polyamine deficiency results in a cell cycle distribution that is similar to that which characterizes the particular cell type, when it is in a state of general nutritional depletion (77, 78, 161). However, the cell cycle distribution that developed during polyamine starvation of CHO-A7 cells was quite different from that occurring following general nutritional depletion. Thus when CHO-A7 cells were grown to a high density, in the presence of

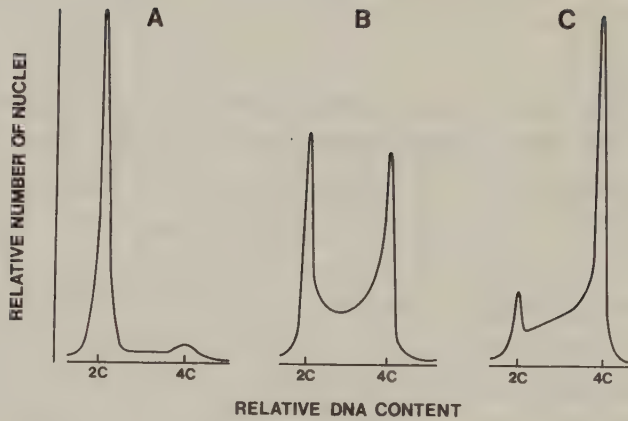


FIGURE 8. DNA distributions of CHO-A7 cells subjected to extended (10 days) ornithine and polyamine starvation. Cells were grown in putrescine-free growth medium containing 0.1 mM ornithine (A); in ornithine- and putrescine-free growth medium (B); or in ornithine- and putrescine-free growth medium supplemented with 1 mM DFMO (C).

Table 2. Effect of extended ornithine and polyamine starvation on cell cycle phase progression of CHO-A7 cells.

Supplements ^a		Phase fractions (%) ^b		
Ornithine	DFMO	G1	S	G2
+	-	95.5	1.5	3
-	-	28.1	41.4	30.5
-	+	10.2	42.4	47.4

^a Cells were grown for 10 days in putrescine-free growth medium (see Section 3.2.2) in the absence/presence of ornithine (0.1 mM) or DFMO (1 mM).

^b Cell cycle phase fractions were derived from the DNA histograms shown in Fig. 8 essentially as described by Dean (135).

ornithine and putrescine, they accumulated in G0/early G1. In fact, 95% of the cells were in G0/early G1 by Day 6 (II).

ELD cells were also found to accumulate in the S and G2 phases during treatment with DFMO, whereas they tended to accumulate in the G1 phase when subjected to general nutritional depletion (i.e. growth to a high cell density) (Table 3). The extent of accumulation in G1 was considerably less for ELD cells than for CHO-A7 cells (cf. Table 2 and 3).

Table 3. Cell cycle distribution of ELD cells at various times of growth - in the absence or presence of DFMO.

Time (hr)	Treatment ^a	Phase fractions (%) ^b		
		G1	S	G2
24	-	33.9	41.8	24.3
48	-	44.2	32.1	23.8
72 ^c	-	50	40.4	9.6
72 ^c	DFMO	36.7	49.1	14.2
96	-	56.1	28.1	15.8
96	DFMO	34.9	33.8	31.4

^a Cells were grown in putrescine-free growth medium (a 1:1 mixture of Ham's F12 and Eagle's MEM, with the various supplements described in Section 3.2.1). DFMO (5 mM) was added at the time of seeding.

^b Cell cycle phase fractions were derived from DNA histograms (obtained by flow cytometric analysis), essentially as described by Dean (135).

^c From reference 162.

The accumulation of cells in the S and G2 phases during polyamine depletion, suggests that the polyamines have a specific function in DNA replication and are not merely nutrients. The depletion of polyamines (putrescine and spermidine) appears to exert a greater inhibitory effect on DNA chain elongation than on DNA chain initiation, since the cells were capable of entering the S phase. That the progression of cells through the S phase was prolonged in polyamine-starved CHO-A7 cells, may also suggest a role for the polyamines in DNA chain elongation. It should be emphasized, however, that further experiments are required to more specifically define the role of polyamines in DNA synthesis.

Sunkara et al. (163) have proposed, that normal cells become arrested mainly in the G1 phase upon polyamine depletion, whereas transformed cells become arrested in the S phase. If this was generally true, it should be possible to accumulate tumor cells in the S phase by DFMO treatment, and to subsequently kill these cells selectively by using S phase specific drugs. Normal cells should be basically resistant to treatment, since they accumulate in G1. Unfortunately, many exceptions have been recorded. Thus, polyamine depletion was found to cause an accumulation of 9L rat brain tumor cells in the G1 phase (77, 153) and CHO cells in the G1 and S phases (164, 165).

Pohjanpelto and Knuutila (166) have shown that the occurrence of chromosome aberrations increases in polyamine-starved cultures of CHO-A7 cells. It has also been shown, that the actin filaments and microtubules disappear upon polyamine starvation (71). The chromosomal defects together with the disappearance of proteins important in cytokinesis, may explain the increased accumulation of multinucleated cells found in polyamine-depleted cultures (71, 160, 165).

Since mycoplasmas may induce chromosome aberrations (167), that are similar in appearance to those observed in polyamine-starved CHO-A7 cells (166), it is obvious that all cell cultures must be mycoplasma-free. The recent finding of Kamatani et al. (168) demonstrates the necessity to work with mycoplasma-free cultures, particularly when studying the stimulating effect of exogenous putrescine on cell growth. Thus, putrescine was found to stimulate mouse T lymphoma cell growth by inhibiting the proliferation of a *Mycoplasma orale* contaminant that uses arginine as a major energy source (168). The antimycoplasmal effects of putrescine prevented the depletion of arginine from the medium. Consequently, the reduction in cellular growth rate was the result of arginine depletion (reduced protein synthesis) and not polyamine depletion (168). Therefore, when attempting to establish polyamine-dependent cell lines, it is important to bear in mind that mycoplasma infection is one mechanism that, under certain conditions, may render mammalian cell lines dependent upon exogenous putrescine for growth. The ornithine and polyamine dependence observed in CHO-A7 cells was clearly not a result of mycoplasma infection, because these cells showed no evidence of such contamination, as determined by three independent techniques (II).

3.6 INTRACELLULAR LOCALIZATION OF ODC

ODC constitutes a very small fraction of the cellular protein, even after maximal enzyme induction (169). Therefore, an ODC overproducing CHO cell mutant (C51.19) was used to study the intracellular localization of ODC (III). The intracellular localization of the enzyme did not show a consistent pattern. The immunocytochemical method indicated that there was a much higher concentration of ODC protein in the cytoplasm than in the nucleus (III). A similar ODC distribution was also observed in proximal tubule cells of the mouse renal cortex, using the same method (138). This localization of ODC is in accordance with the biochemical studies, which indicate that ODC is mainly a cytosolic enzyme (91, 92).

At variance with these findings, autoradiographic analyses of ^3H -DFMO binding indicated, that the concentration of catalytically active ODC was roughly similar in the nucleus and the cytoplasm (III). This ODC distribution pattern is similar to that observed in polychaete germ cells (95, 96) and in proximal tubule cells of the mouse renal cortex (170), using the same method of analysis.

There are at least two possibilities that may explain the differences between the immunocytochemical and the autoradiographic data. 1) Since the ODC antibody was raised against a preparation of homogeneous ODC, which was cytosolic in origin, it is conceivable that the antibody exhibits a higher affinity for cytoplasmic ODC. Obviously, this idea is based on the assumption that there are different forms of ODC being preferentially associated with either the cytoplasm or the nucleus. Evidence for posttranslational ODC modification has been presented (97, 171), but this area requires further experimental analysis before any firm conclusions can be drawn. 2) The two methods in fact do not demonstrate the same thing. Thus, the immunocytochemical method shows the distribution of ODC protein (possibly including catalytically inactive ODC), whereas the autoradiographic methods show the distribution of catalytically active ODC. This might suggest the presence of a cytoplasmic store of catalytically inactive ODC, either a precursor molecule that might be activated by cleavage, or a posttranslationally modified enzyme that may or may not have the capacity to become reactivated.

Even though the immunocytochemical method indicated a predominantly cytoplasmic localization of ODC, the possibility of a nuclear localization of the enzyme cannot be excluded. In fact, preliminary studies

show a high fluorescence intensity in some nuclei of partly synchronized cells.

3.7 POLYAMINES AS TUMOR MARKERS - RESULTS AND DISCUSSION

3.7.1 Urinary Polyamine Excretion - Experimental Considerations

To be able to relate our experimental data to other studies (including clinical ones), all urine samples were hydrolyzed before analysis (IV; V; VI). This procedure yields an estimate of the total amount of polyamines, i.e. free and conjugated. Although recent analytical developments have made it possible to analyze physiological fluids for their free and conjugated polyamines, we have not included such analyses, for the reason mentioned above.

A large number of polyamine derivatives have been identified (29). For example, N¹-monoacetylputrescine, N¹-monoacetylspermidine and N⁸-monoacetylspermidine were found to be the major polyamine derivatives excreted in cancer patients (172, 173). In fact, about 80% of the urinary spermidine was excreted as acetyl derivatives. Furthermore, the increase in urinary polyamine excretion associated with cancer, was attributable to acetylated polyamines rather than free polyamines (172, 173). It was also postulated that the ratio of N¹-monoacetylspermidine to N⁸-monoacetylspermidine might be a better indicator of tumor burden than the total polyamine excretion. Another study, however, indicates that the determination of this ratio may be of only limited value as an indicator for the presence of tumors (174).

3.7.2 Urinary Polyamine Excretion - As Related to Tumor Growth and Regression

In tumor-bearing rats, the 24-hr urinary putrescine excretion was found to increase with increasing tumor burden (IV; V). However, the tumor had to reach a certain size (about 2 cm³) before any changes in putrescine excretion could be detected (IV; V). The spermidine excretion remained constant throughout the experiments, as did that of creatinine (IV; V). Spermine was barely detectable in the 24-hr urines (IV; V).

There was an earlier and greater increase in putrescine excretion when the NG-W1 tumor was grown intrahepatically than when it was grown subcutaneously (Fig. 9) (IV). This was true even though these tumors showed roughly the same growth rate and reached almost the same size

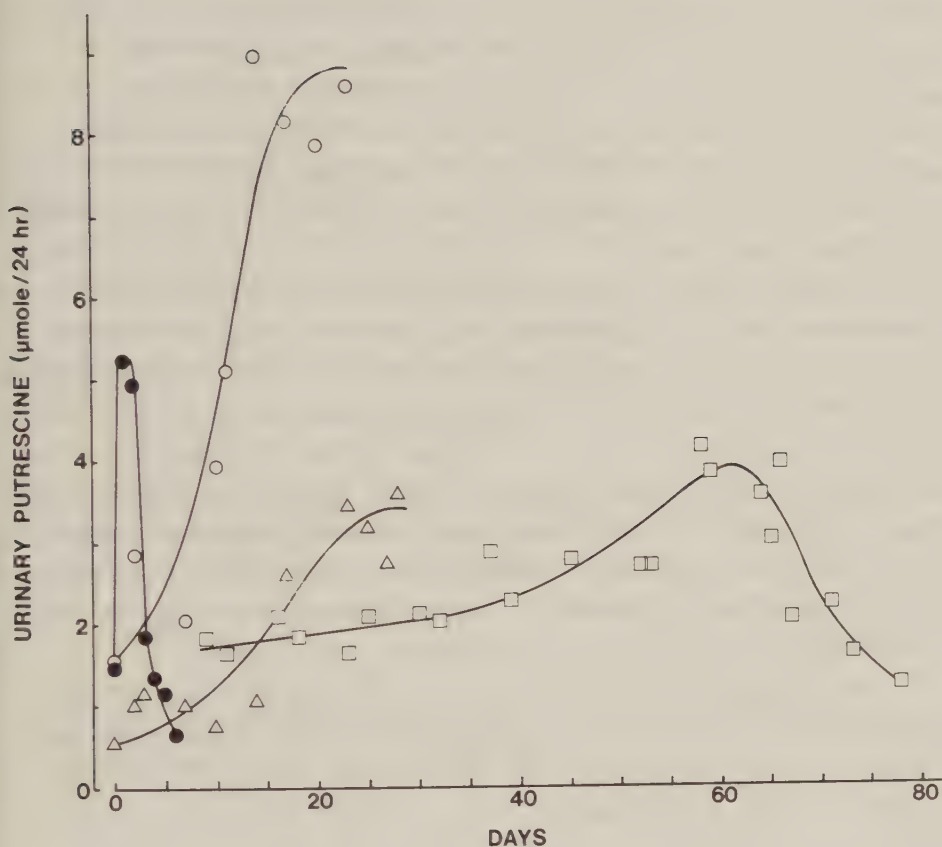


FIGURE 9. Twenty-four-hr urinary putrescine excretion following intra-hepatic transplantation of NG-W1 adenocarcinoma (○); s.c. transplantation of NG-W1 adenocarcinoma (Δ); DMBA induction of mammary carcinomas (□); and CCl₄ induction of liver necrosis (●). Data points shown are those obtained for one representative rat in each experimental system.

during the initial 14 days of growth (IV). A probable explanation for this earlier and greater increase in putrescine excretion, is that the tumor develops a much better vascularization when grown intrahepatically (120).

As a means of studying the effect of tumor regression on polyamine excretion, an estrogen-dependent mammary carcinoma was used (V). Bilateral ovariectomy produced a rapid decrease in tumor mass paralleled by a decrease in 24-hr urinary putrescine excretion (V). Unexpectedly, there was a decrease in putrescine excretion even in non-ovariectomized rats (Fig. 9) (V). This occurred almost in parallel with the decrease

observed in ovariectomized rats (V). A possible explanation for this apparent contradiction is that tumor progression causes a state of overt malnutrition, involving complex disturbances of the host metabolism. This is shown for example by the fact that the body weight decreased in rats with advanced tumors. Furthermore, the 24-hr urinary excretion of spermidine decreased in parallel with that of putrescine, as did the urine volume.

The present data suggest that the increased urinary putrescine excretion in tumor-bearing animals is a function of the tumor burden rather than the growth rate of the tumor. Since spermidine and spermine exhibited no such relationship, putrescine appears to be the only polyamine that is potentially useful as a tumor marker. Similar conclusions have been drawn from other experimental tumor systems and from clinical studies (113, 114, 175-177). In patients with medulloblastoma, putrescine was the most consistently elevated polyamine, although spermidine was also significantly elevated at times (112-114). Spermine, however, was present only in occasional cerebrospinal fluid samples.

Since tumor growth is a process that includes both cell proliferation and cell death, the increased urinary putrescine excretion may be either a result of release from proliferating cells (102) or from dead or dying cells (103, 104).

3.7.3 Increased Urinary Polyamine Excretion - A Function of Cell Proliferation or Cell Death?

To reveal whether the 24-hr urinary putrescine excretion increases as a function of cell proliferation or cell death, CCl_4 intoxication of the rat was used as an experimental model (VI). Treatment with CCl_4 produces an initial phase of liver cell death, succeeded by a regenerative phase of growth. The highest rate of putrescine excretion occurred during the first 24 hr of CCl_4 intoxication (Fig. 9), and coincided with the period of maximal liver damage. During subsequent liver regeneration, as demonstrated by ^3H -thymidine incorporation into liver DNA, putrescine excretion decreased. These results indicate that the urinary putrescine excretion increases as a function of cell death rather than cell proliferation. The spermidine excretion showed the same pattern of changes as did that of putrescine, although the changes were much less pronounced (VI). Spermine was barely detectable in the 24-hr urines (VI).

The increased rate of urinary putrescine excretion during the

period of liver cell death, may be partly attributable to the dramatic increase observed in liver putrescine concentration (34, 178, 179). This accumulation of putrescine is a function of both increased ODC activity (putrescine synthesis) and increased spermidine/spermine N^1 -acetyltransferase activity (conversion of spermine and spermidine into N^1 -monoacetylputrescine). Theoretically, the acetylated polyamines can more easily cross the cell membrane (180). In addition to the possibility that acetylated polyamines are transported across the hepatocyte membrane at an increased rate, changes in the membrane caused by CCl_4 treatment may lead to an increased leakage of polyamines (and other cellular components) from the cells into the circulation.

3.7.4 Physiological Fluid Polyamines - Markers of Tumor Cell Death

Taken together, our results indicate that putrescine can be used as a marker of tumor burden and that the increase in putrescine excretion is coupled to tumor cell death.

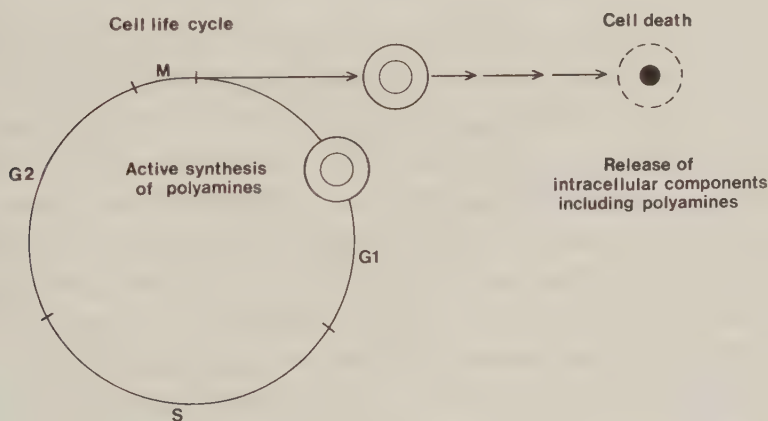


FIGURE 10. Polyamine synthesis and excretion as related to cell growth and cell death.

As a result of cell death, which accompanies growth and proliferation of a tumor, there should be an increased release of not only polyamines, but also other intracellular components (Fig. 10). Accordingly, we have observed an increased excretion of osmotically active substances, such as urea, during tumor growth (Anehus, Yngner, Hafström & Heby, unpubl.). This may explain the increase in 24-hr urine volume

observed to parallel the increase in tumor burden (IV; V). The fact that there was a high positive correlation between the 24-hr urine volume and the 24-hr urinary putrescine excretion, raised the question whether the polyamines themselves might exert a diuretic effect, as suggested in a preliminary note (181). No evidence to this effect was obtained, however, when putrescine or spermidine were injected intraperitoneally into rats.

A large body of evidence suggests that analyses of physiological fluid polyamines may serve as a diagnostic aid, together with established techniques, in cases of suspected cancer. The problems associated with collecting 24-hr urines may be circumvented by using early morning urines, since their polyamine contents apparently change according to the same pattern as do 24-hr urinary polyamine content (182). Furthermore, since the amount of creatinine excreted remained constant during tumor growth (IV; V), polyamine excretion may be expressed on a per mg creatinine basis.

3.8 SUMMARY

The role of the polyamines in cell growth and division has been studied by analyzing the effects of cellular polyamine depletion. Thus, arginase-deficient Chinese hamster ovary (CHO-A7) cells were starved for ornithine and polyamines, and Ehrlich ascites tumor (ELD) cells were treated with α -difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase (ODC). As a result, the cellular putrescine and spermidine content decreased to non-detectable levels, whereas the spermine content increased. Cellular putrescine and spermidine depletion caused a marked reduction in growth rate, attributable to an accumulation of cells in the S and G2 phases of the cell cycle. The progression of cells through the S phase was slowed, but did not come to a complete arrest. These experiments indicate that the polyamines play a role in DNA replication, probably by participating as cofactors in the DNA synthetic reaction. That cells accumulated in the G2 phase upon prolonged polyamine deficiency may be a result of accumulation of errors in DNA during replication, which make the cells incapable of going through mitosis.

These results may be taken to denote that the polyamines and/or their biosynthetic enzymes have a regulatory function within the cell

nucleus. Therefore, it seemed appropriate to determine whether ODC is entirely cytoplasmic in its localization, as previously suggested. Since ODC constitutes a very small fraction of the cellular protein, even after maximal enzyme induction, an ODC overproducing Chinese hamster ovary cell mutant was used. Indirect immunofluorescence microscopy, using a monospecific ODC antibody, suggested a predominantly cytoplasmic localization of ODC, whereas light and electron microscope autoradiography, using ^3H -DFMO, indicated an equal distribution between the nucleus and the cytoplasm. The data indicate that ODC is present in the nucleus, but that the amount of ODC protein and ODC-catalytic activity may vary.

The 24-hr urinary excretion of polyamines was studied in relation to tumor growth and regression of a transplantable adenocarcinoma and a hormone-dependent mammary carcinoma of the rat. The urinary putrescine excretion was found to increase with increasing tumor burden, whereas the spermidine excretion remained constant. Spermine was barely detectable. There was an earlier and greater increase in putrescine excretion when the adenocarcinoma was grown intrahepatically than when it was grown subcutaneously. A probable explanation for this finding is that the tumor develops a much better vascularization when grown intrahepatically. Bilateral ovariectomy induced a rapid regression of the estrogen-dependent mammary carcinoma paralleled by a decrease in urinary putrescine excretion. Whether the excretion of urinary putrescine increases as a function of cell proliferation or cell death was studied, using CCl_4 intoxication of the rat as an experimental model. The highest rate of urinary putrescine excretion was observed during the period of maximal liver necrosis, whereas the putrescine excretion decreased during liver regeneration. These results indicate that putrescine can be used as a marker of tumor burden and that the increase in putrescine excretion is coupled to tumor cell death.

4. ACKNOWLEDGEMENTS

This work has been carried out at the Department of Zoophysiology, University of Lund, Sweden.

I owe deep gratitude to Olle Heby, my teacher, for introducing me to the field of polyamine research and for continual support and encouragement.

I also wish to express my warm thanks to:

Margareta Linden, Stina Oredsson and Bertil Löwkvist, my friends and collaborators in the polyamine group;

my other collaborators who have participated in various aspects of the work;

Marianne Andersson for invaluable help with art work and typing;

Rikard, my husband, for his never ending support and understanding.

The major part of this work was supported by grants from the Swedish Natural Science Research Council. Subsidiary support was received from the Medical Faculty (University of Lund), the Segerfalk Foundation, the John and Augusta Persson Foundation and the Sigrid Jusélius Foundation, the Swedish Medical Research Council and the Royal Physiographical Society (Lund).

DL- α -methylornithine monohydrochloride monohydrate (RMI 71,633-A10) and DL- α -difluoromethylornithine monohydrochloride monohydrate (RMI 71,782-A25) were generous gifts from Centre de Recherche Merrell International, Strasbourg, France.

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Localization of Ornithine Decarboxylase in Mutant CHO Cells That Overproduce the Enzyme.

Differences Between the Intracellular Distribution of Monospecific Ornithine Decarboxylase Antibodies and Radiolabeled α -Difluoromethyl-ornithine.

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RUNNING TITLE: INTRACELLULAR LOCALIZATION OF ORNITHINE DECARBOXYLASE.

SUMMARY

The intracellular localization of ornithine decarboxylase (ODC), a key enzyme in polyamine synthesis and cell growth, is a matter of present debate. Using two independent methods of analysis, we have attempted to determine the actual distribution of ODC in a mammalian cell. To overcome the problem of a normally very low cellular ODC content, we have used an ODC overproducing mutant of Chinese hamster ovary (CHO) cells. These mutant cells exhibit a 10-fold higher ODC activity than do the wild-type cells. The localization of ODC protein in exponentially growing cells, was determined by indirect immunofluorescence microscopy (permeabilized cells), using monospecific ODC antibodies. The intracellular localization of catalytically active ODC was determined by light and electron microscope autoradiography following pulse-labeling of cells with α -difluoromethyl(5- ^3H)ornithine (^3H -DFMO) at the time of peak ODC activity. α -Difluoromethylornithine (DFMO) is an enzyme-activated irreversible inhibitor of ODC and binds covalently to the active enzyme. The specificity of this reaction in the cell was ascertained by immunoprecipitation of ^3H -DFMO-labeled ODC. ODC (as determined by both methods) was present in all the cells of a serum-stimulated monolayer culture. The highest concentration of ODC protein and of catalytically active ODC was observed in the smallest and most rapidly proliferating cells. Polyploid and multinuclear cells always exhibited the lowest concentrations. As to the intracellular localization, the immunocytochemical method indicated that there was a much higher concentration of ODC protein in the cytoplasm than in the nucleus, whereas the autoradiographic methods indicated that the concentration of catalytically active ODC was similar in the nucleus and the cytoplasm.

INTRODUCTION

ODC, which is a rate-controlling enzyme in polyamine biosynthesis, has been shown to play an important role in cell growth and differentiation (reviewed by Heby, 1981). The intracellular localization of ODC, was first studied biochemically by assaying for its activity in subcellular fractions. These experiments suggested that ODC was predominantly or exclusively a cytoplasmic enzyme, at least in growth-stimulated mouse fibroblasts and in rat liver and prostate (Grillo & Fossa, 1983; McCormick, 1977; Murphy & Brosnan, 1976; Ono et al., 1972; Pegg & Williams-Ashman, 1968). Using another fractionation technique, Bartho-leyns (1983) observed a different subcellular ODC distribution pattern in the unstimulated rat liver; 40% of the activity was nuclear (mainly nucleolar) and 43% was cytoplasmic. After growth-stimulation, however, there was a marked increase in the activity of cytoplasmic ODC. A recent biochemical study on barley seeds even indicates that the ODC activity is localized mainly in the nucleus, tightly bound to chromatin (Panagiotidis et al., 1982). Furthermore, the proposal that ODC after either phosphorylation (Atmar & Kuehn, 1981) or transglutamination (Russell, 1983) may serve as an activator of RNA polymerase I, is consistent with a nuclear localization for at least part of the ODC protein.

The synthesis of DFMO (Metcalf et al., 1978), and its tritium-labeled derivative (^3H -DFMO), and the isolation of monospecific ODC antibodies (Persson, 1982), for the first time made it possible to study the intracellular localization of ODC by means of microscopic techniques. DFMO binds specifically to the active site of ODC (Metcalf et al., 1978), thus indicating catalytically active enzyme, whereas the monospecific antibody binds to ODC protein, including catalytically inactive ODC, thus indicating total enzyme protein.

In studies of the intracellular localization of the sites of irreversible binding of ^3H -DFMO to the active enzyme by light and electron microscope autoradiography, it was demonstrated that ODC is present at a high concentration not only in the cytoplasm but also in the nucleolus and the nucleoplasm of female germ cells of a polychaete (Emanuelsson & Heby 1982; 1983). In apparent contrast, immunocytochemical analyses of proximal tubule cells of the mouse renal cortex suggest

a predominant cytoplasmic localization of ODC (Persson et al., 1982).

The aim of the present study, was to determine whether the ODC distribution pattern obtained by ^3H -DFMO-autoradiography differs from that obtained by immunofluorescence, when using the same cell material. Due to the very small amount of ODC normally present in cells, a somatic cell mutant (CHO-C51.19) that overproduces the enzyme (Choi & Scheffler, 1981) was used for this comparison. Other variants exhibiting elevated ODC activities have been isolated from nonmutagenized populations of rat hepatoma (Mamont et al., 1978b) and mouse lymphoma (McConlogue & Coffino, 1983a, b). However, these variants were considered less suitable for this particular study, since they grow in suspension cultures and thus do not spread out to facilitate intracellular distribution analysis. The ODC distribution patterns obtained by the autoradiographic and immunofluorescent techniques indeed proved to be different. Possible explanations for this difference are discussed.

MATERIALS AND METHODS

Cell line. In this investigation we used an ODC overproducing CHO cell mutant (C51.19), that was isolated following mutagenesis (ethylmethane sulfonate, 400 $\mu\text{g/ml}$, 24 h) of several million wild-type cells (CHO-Toronto) (Choi & Scheffler, 1981). After a few days of growth in the absence of the drug, α -methylornithine (MO), a competitive and reversible inhibitor of ODC (Bey et al., 1978), was added to the medium. The small number of resistant clones growing in the presence of 8.5 mM MO was isolated and maintained in medium containing 10 mM MO. One of these clones (C51) was subjected to further selection in the presence of 0.85 mM DFMO, the enzyme-activated and irreversible inhibitor of ODC. Several resistant clones were isolated. Resistance to MO and DFMO was found to be associated with ODC overproduction (Choi & Scheffler, 1981). Some clones, including C51.19, exhibited 10-fold higher levels of induced ODC activity than the parental CHO cells. The resistant phenotype of these mutant CHO cells is stable under nonselective conditions. The enzyme itself is apparently unaltered by the mutation (Choi & Scheffler, 1981).

Routine cultivation. The cells were maintained in 50 mm plastic

petri dishes (Nunc) in the following growth medium: Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum (Gibco), 1 mM proline (Fluka), penicillin (50 IU/ml)-streptomycin (50 µg/ml) mixture (Gibco) and 44 mM NaHCO₃ (pH 7.3). The cultures were incubated at 37°C in a high-humidity atmosphere of 5% CO₂ in air. The cells were subcultivated every 3 to 4 days (1:20 - 1:70 split).

Experimental growth conditions. Cells were plated in 35, 50 or 90 mm plastic petri dishes (2.5×10^4 cells/cm²) in high-serum (10%) growth medium and grown in the absence or presence of 10 mM MO for 48 h. They were rinsed with serum-free growth medium and then supplied with low-serum (0.1%) growth medium. After 16 h of serum starvation, the cells were stimulated to grow by adding high-serum (10%) growth medium. Maximum ODC levels were observed 5 h later (Choi & Scheffler, 1981).

For Nomarski differential interference contrast and immunofluorescence microscopy, cells were grown on glass coverslips.

Immunofluorescence microscopy. 5×10^5 cells were plated in 50 mm plastic petri dishes containing glass coverslips and were grown as described in "Experimental growth conditions". Five hours after serum stimulation, the cells were fixed in a solution of 4% paraformaldehyde and 5 mM dithiothreitol in Tyrode's balanced salt solution (pH 7.2) for 1 h at 4°C. Dithiothreitol stabilizes ODC (Jänne & Williams-Ashman, 1971) and appears to enhance immunoreactivity of the enzyme (Persson et al., 1982). After fixation, the cells were rinsed thoroughly in Tyrode's balanced salt solution containing 10% sucrose (pH 7.2, 4°C) and were then stored at -20°C.

The cells were processed for indirect immunofluorescence microscopy mainly as described by Coons et al. (1955). They were rendered permeable to the antibodies by preparing all solutions in permeabilizing buffer (0.25% Triton X-100 and 0.25% human serum albumin in phosphate-buffered saline (PBS)). The monospecific ODC antiserum used has been previously documented (Persson, 1982). It was prepared by immunizing rabbits with a mixture of homogeneous mouse kidney ODC and Freund's complete adjuvant. The fixed cells were incubated with ODC antiserum, diluted 1:640, for 3 h at room temperature and thoroughly rinsed. The sites of the antigen-antibody reaction in the cells were then visualized by incubation with fluorescein isothiocyanate (FITC)-conjugated goat

anti-rabbit IgG (DAKO, Copenhagen, Denmark). The second antibody, diluted 1:20, was applied for 30 min at room temperature. The cells were finally rinsed and mounted in PBS-glycerol (1:1).

Specificity controls were run according to Sternberger (1974). They consisted of comparison of fluorescence of cells exposed to (A) inactivated (incubated with homogeneous mouse kidney ODC; 100 $\mu\text{g/ml}$ of diluted antiserum) ODC antiserum and (B) ODC-unrelated (anti-gastrin) antiserum. Cells were also incubated with FITC-conjugated goat anti-rabbit IgG only (without a primary antibody) and examined for positive fluorescence.

Binding of ^3H -DFMO to ODC for autoradiography. 7.5×10^4 cells were plated in 35 mm plastic petri dishes (either on glass slide pieces or directly on the plastic substrate) as described in "Experimental growth conditions". One hour after serum stimulation, 2.78 MBq of ^3H -DFMO (specific activity, 636.4 GBq/mmol; New England Nuclear) was added to the growth medium (1.5 ml), yielding a 2.9 μM concentration. The cells were grown in the presence of ^3H -DFMO for 4 h. They were then subjected to a chase for 0, 15, 30 or 60 min in 5 ml of high-serum growth medium supplemented with 10 mM DFMO (unlabeled). The cells were finally fixed in 2% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.3) for 1 h at 4°C.

Autoradiography. The glutaraldehyde-fixed cells were post-fixed in a solution of 1% osmium tetroxide in 0.2 M cacodylate buffer (pH 7.3) for 1 h at 4°C. Following rinsing in cacodylate buffer, the cells were dehydrated and stained in ethanol (90%) containing 1% phosphotungstic acid and 0.5% uranyl acetate. Cells, fixed directly on the plastic substrate, were rinsed with 90, 95 and 97% 2-hydroxypropyl methacrylate (HPMA) in water and finally with a 1:1 mixture of HPMA and Epon 812. The cells were embedded in Epon 812, detached from the plastic/glass substrate and sectioned for light and electron microscope autoradiography.

The 1 μm sections, for light microscope autoradiography, were coated with Ilford K 2 liquid nuclear emulsion, according to the dipping method, and exposed for 2-3 weeks at 4°C. The autoradiographs were developed in Kodak D 19 (5 min, 18°C), briefly rinsed in distilled water and fixed in Kodak F 24 (6 min, 18°C). They were finally stained in a solution containing Richardson's azure II and methylene blue. The distribution of ^3H -DFMO-labeling was quantified by visual counts of

silver grains in the emulsions at 400x magnification. Background counts were subtracted.

The ultrathin sections, for electron microscope autoradiography, were first coated with a protective carbon layer and then with Ilford L 4 liquid nuclear emulsion, according to the loop method. After exposure for 1-3 months at 4°C, the autoradiographs were developed in Kodak D 19 (2 min, 20°C), rinsed in distilled water (30 sec), fixed in newly made 15% Na₂S₂O₃ (3 min, 20°C) and finally washed in distilled water. They were examined in a Jeol 100 CX electron microscope at the Unit of Electron Microscopy, Department of Zoology, University of Lund.

Binding of ³H-DFMO to ODC for immunoprecipitation. 5x10⁵ cells were plated in 50 mm plastic petri dishes and were grown as described in "Experimental growth conditions". One hour after serum stimulation, 1.85 MBq of ³H-DFMO (specific activity, 636.4 GBq/mmol) was added to the growth medium (1 ml), yielding a 2.9 μM concentration. The cells were grown in the presence of ³H-DFMO for 4 h. They were then subjected to a chase for 0, 30 or 60 min in 5 ml of high-serum growth medium supplemented with 10 mM DFMO (unlabeled). The cells were finally trypsinized (0.25% trypsin and 0.02% Na₂EDTA in PBS; 37°C, 5 min), suspended in high-serum growth medium and collected by centrifugation.

Immunoprecipitation. About 2x10⁶ ³H-DFMO-treated cells were sonicated in 100 μl of Buffer A: 0.1 M Tris-HCl buffer (pH 7.5) containing 0.1 mM Na₂EDTA and 2.5 mM dithiothreitol. A 100 μl aliquot of the 45,000 xg (1 h) supernatant (approx. 14 nmol of CO₂/h x 100 μl) was allowed to react with 400 μl of ODC antiserum (1:40 or 1:80 dilution in Buffer A; titer: 600 nmoles of CO₂/h x μl) (Persson, 1982) for 24 h in the cold. Then 200 μl of goat anti-rabbit serum (undiluted or a 1:1 dilution in Buffer A) was added. After 24 h in the cold, the samples was mixed with 500 μl of Buffer A. The immunoprecipitate was collected by centrifugation at 3000 xg for 30 min and dissolved in 500 μl of Soluene 350 (Packard). The supernatant was centrifuged at 45,000 xg for 1 h. The proteins contained in the 45,000 xg supernatant were precipitated by the addition of 4% sulfosalicylic acid. The precipitate was washed 5 times by sonication in 4% sulfosalicylic acid and finally dissolved in 500 μl of Soluene 350.

Precipitates, dissolved in Soluene 350, were mixed with 10 ml of Permablend III (Packard) (5.5 g/l of toluene) and counted in an LKB-

-Wallac 1217 Rackbeta Liquid Scintillation Counter.

ODC-assay. All experimental protocols contained parallel cultures for monitoring of the ODC activity. The cells were disrupted by sonication in 10 mM Tris-HCl buffer (pH 7.2) containing 0.5 mM Na₂EDTA, 5 mM dithiothreitol and 0.05 mM pyridoxal 5'-phosphate. The ODC activity was assayed for in the presence of a saturating (1 mM) concentration of ornithine, mainly as described by Heby et al. (1975).

Polyamine analysis. For polyamine analysis, the cells were disrupted by sonication in ice-cold 0.2 M perchloric acid. The homogenates were kept on ice for 1 h and were then centrifuged at 1000 xg for 10 min. The supernatant was analyzed for its polyamine content, using a thin-layer chromatographic (TLC) method (Seiler, 1970), essentially as described by Heby et al. (1973). In the present study, however, the TLC plates were not sprayed with the mixture that was previously used to increase and stabilize the fluorescence. Better reproducibility and sufficient sensitivity was achieved without spraying. The TLC plates were dried for about 30 min and analyzed on an Aminco-Bowman spectro-photofluorometer equipped with a TLC-scanner.

RESULTS

Characterization of the ODC overproducing cells. Mutant CHO cells that overproduce ODC grow in culture with a population doubling time (T_D = 17-18 h) that is significantly longer than that of wild-type CHO cells (T_D = 12-14 h). A majority of the mutant cells are small, spheroidal and somewhat flattened (Fig. 1). In addition to these cells, a low frequency of giant, flat cells, which are polyploid and/or multinucleate - some with as many as 25 nuclei, appear among the mutant CHO cells. These giant cells are present both during normal growth conditions and during MO treatment. Mutant CHO cells, like their wild-type counterparts, do not form a regular monolayer when growing to a high density. In fact they begin to pile up on each other already in relatively sparse cultures.

The cell line used (C51.19) exhibits about a 10-fold higher ODC level than the parental cells (Choi & Scheffler, 1981). To achieve maximal ODC activity, the mutant cells were subjected to a 48 h pulse

of MO during the growth phase immediately prior to serum starvation. MO treatment caused an 8-fold elevation of the basal ODC levels observable after 16-24 h of serum starvation, and a 4-fold higher level of ODC activity upon serum stimulation (Choi & Scheffler, 1981). The overproduction of ODC in mutant CHO cells caused a considerable increase in their polyamine content as compared to wild-type cells (cf. Table 1 with Steglich & Scheffler, 1982). There was a 15-fold increase in putrescine content but a more moderate (2.4 - 5.9-fold increase in spermidine and spermine content (Table 1).

Localization of ODC in ODC overproducing cells by immunofluorescence microscopy. Figure 2 shows the distribution of immunoreactive ODC protein in a whole-cell preparation of ODC overproducing mutant CHO cells. All cells were immunoreactive, but small cells always exhibited more intense fluorescence than did large cells. MO-treated cells exhibited somewhat more intense fluorescence than did untreated cells, but there was no difference in terms of the intracellular distribution (not shown). The fluorescence pattern, which was basically the same in all cells, suggests that the majority of the enzyme protein is localized in the cytoplasm - the perinuclear region always being most intensely stained. In metaphase cells, chromosomes appear as dark areas, suggesting very small amounts of chromatin-associated ODC (not shown). The interphase cell nucleus and the mitotic chromatin exhibited some fluorescence, but since whole cells were analyzed we cannot exclude the possibility that it was due to the presence of cytoplasm above and below the nucleus/chromatin (Fig. 2). At this point it should be emphasized that all controls were negative, and most importantly, that all immunoreactivity in the cells was abolished by absorbing the antibodies with homogeneous ODC.

Localization of ODC in ODC overproducing cells by light and electron microscope autoradiography. Figures 3 and 4 show the distribution of catalytically active ODC in sections (1 μ m - ultrathin) of ODC overproducing mutant CHO cells. Silver grains, indicating ^3H -DFMO covalently bound to ODC, were distributed fairly evenly throughout the cells. The grain density was roughly similar in the nucleus and in the cytoplasm. The number of grains observed over the nucleus constituted between 40 and 60% of the total number over the cell. MO-treated cells contained a somewhat greater number of silver grains

than did untreated cells, but there was no difference in terms of their intracellular distribution (not shown). At the exposure times used for autoradiography, the background grain count was negligible. A 15-60 min chase with cold DFMO did not significantly affect the total grain count or its distribution.

Identification of ^3H -DFMO-bound ODC by immunoprecipitation. The specificity of the ^3H -DFMO-binding was determined by immunoprecipitation of ODC protein with a monospecific antibody as shown in the flow diagram (Fig. 5). The ODC antibody precipitated 95.9% of the radioactivity associated with soluble cell protein. This precipitate constituted an exceedingly small part of the total soluble protein content.

DISCUSSION

ODC is a key enzyme in polyamine biosynthesis and cell growth (reviewed by Heby, 1981). Thus, lack of ODC activity, because of a mutated gene (Steglich & Scheffler, 1982) or treatment with specific inhibitors (Mamont et al., 1978a; Metcalf et al., 1978; Danzin et al., 1983) results in polyamine deficiency and growth inhibition. A majority of studies on the intracellular distribution of ODC suggest that ODC is localized and functions predominantly or exclusively in the cytoplasm (Grillo & Frossa, 1983; McCormick, 1977; Murphy & Brosnan, 1976; Ono et al., 1972; Pegg & Williams-Ashman, 1968). Recent evidence, however, biochemical as well as autoradiographic, indicates that ODC is present in the nucleus at a significant level (Atmar & Kuehn, 1981; Emanuelsson & Heby, 1982; 1983; Panagiotidis et al., 1982; Zagon et al., 1983). Some reports even indicate that the nuclear ODC activity may considerably exceed that of the cytoplasmic (Panagiotidis et al., 1982). Conceivably, shortcomings in the cell fractionation techniques that were used initially, may have confused the issue of intracellular ODC localization much the same way that they did the DNA polymerase localization (Herrick et al., 1976). Thus, DNA polymerase was initially found only in the cytoplasm.

To determine the actual distribution of ODC in a mammalian cell, we used two independent methods of analysis, indirect immunofluorescence microscopy and light and electron microscope autoradiography.

Since the immunocytochemical method depends on the passage of the antibodies across the cell and nuclear membranes and on the specificity of the antibodies, the cells were permeabilized and treated with ODC antibodies with documented high titre and monospecificity.

The validity of ^3H -DFMO experiments is in turn contingent upon the specificity of the inhibitor. DFMO exhibits a very high specificity for ODC because the irreversible inactivation of the enzyme does not occur directly upon binding (Metcalf et al., 1978). First the inhibitor is accepted by ODC as a substrate and then it is enzymatically decarboxylated to generate a highly reactive intermediate. This intermediate alkylates a nucleophilic residue at, or near, the active site of ODC, thus covalently binding the inhibitor to the enzyme (Metcalf et al., 1978). It has also been shown that ODC is inactivated by radiolabeled DFMO in parallel with the incorporation of radioactivity into ODC protein (Pritchard et al., 1981; Seely et al., 1982a; b). This incorporation is highly specific because only a protein of M.W. 53,000, which corresponds to the M.W. of the ODC subunit, was found to be labeled *in vivo* as determined by polyacrylamide gel electrophoresis.

To remove free ^3H -DFMO (not irreversibly bound to ODC), cells were subjected to a chase with unlabeled DFMO (10 mM) for 15, 30 or 60 min. The chase did not alter the distribution of silver grains within the cell, nor did it significantly decrease the total number of silver grains per cell. Therefore, it appears that free ^3H -DFMO is effectively eliminated in the fixation procedures, and not retained in the cells as a result of a reaction with the aldehyde fixative. The possibility that a chase might have eluted free ^3H -DFMO more effectively from the cytoplasm than from the nucleus was also ruled out by these experiments.

Considering that ODC normally has a very short half-life (10-15 min), it may seem surprising that a 60 min chase did not cause a decrease in bound radioactivity. However, the halflife of ODC in mutant CHO cells is markedly prolonged ($t_{1/2}$ = 90-120 min at 4 h after high-serum stimulation) (Choi & Scheffler, 1981). Therefore, even the 60 min chase was not long enough to reveal a significant decrease in bound radioactivity, as demonstrated by others (Seely et al., 1982a). Even though MO has been shown to increase the half-life of ODC in several cell types (McCann et al., 1977), the treatment of mutant CHO cells

with MO, which is part of the present experimental protocol, did not affect the half-life of ODC (Choi & Scheffler, 1981).

The methods used to determine the intracellular localization of ODC, revealed clearly divergent distribution patterns. The immunocytochemical method indicated that there was considerably more ODC in the cytoplasm than in the nucleus, whereas the autoradiographic methods indicated that ODC was evenly distributed in the cell. There are several possible explanations for these differences. The possibility that the ODC antibody did not readily enter the nuclear compartment is ruled out by the fact that permeabilized cells were used in our experiments. The possibility of unspecific binding of ^3H -DFMO to nuclear proteins is in turn ruled out by the fact that almost all (96%) the bound ^3H -DFMO was precipitated by the monospecific ODC antibody. That the ODC antibody used, for immunoprecipitation and for indirect immunofluorescence analysis, was indeed monospecific has been demonstrated by 2-dimensional gel analysis (McConlogue & Coffino, 1983a; Persson et al., submitted).

This leaves us with two further possibilities, and at the present time we cannot exclude either one. Since the ODC antibody was raised against a preparation of homogeneous ODC, which was largely cytoplasmic in origin, it is conceivable that the antibody exhibits a higher affinity for cytoplasmic ODC. Obviously, this idea is based on the assumption that there are different forms of ODC being preferentially associated with either the cytoplasm or the nucleus. Evidence for posttranslational ODC modification has been presented (Atmar & Kuehn, 1981; Russell, 1983), but this area requires further experimental analysis before any firm conclusions can be drawn. The alternate explanation for the observed differences in intracellular distribution of ODC is that the methods in fact do not demonstrate the same thing. The immunocytochemical method shows the distribution of ODC protein (possibly including catalytically inactive ODC), whereas the autoradiographic methods show the distribution of catalytically active ODC. This might suggest the presence of a cytoplasmic store of catalytically inactive ODC, either a precursor molecule that might be activated by cleavage, or a posttranslationally modified enzyme that may or may not have the capacity to become reactivated.

ACKNOWLEDGEMENTS

This investigation was supported by grants from the Swedish Natural Science Research Council, the Swedish Medical Research Council and the Medical Faculty, University of Lund. The excellent technical and secretarial assistance of Annagreta Petersen and Marianne Andersson, respectively, is gratefully acknowledged. DL- α -Methylornithine monohydrochloride monohydrate (RMI 71,633-A10) and DL- α -difluoromethylornithine monohydrochloride monohydrate (RMI 71,782-A25) were generous gifts from Centre de Recherche Merrell International, Strasbourg, France.

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Table 1. ODC activity and polyamine content of ODC-overproducing CHO cells.

Time ^a h	ODC activity ^b Units/10 ⁶ cells ^c	Putrescine	Spermidine	Spermine
		nmoles/10 ⁶ cells ^c		
0	79	6.91	2.07	4.94
5	355	6.86	4.10	5.41

^a The cells were grown in high-serum (10%) growth medium supplemented with 10 mM MO for 48 h. They were then rinsed and supplied with low-serum (0.1%) growth medium for 16 h. The cellular ODC activity and polyamine content were determined at the time of addition of high-serum (10%) growth medium (0 h) and 5 h after serum stimulation.

^b 1 Unit = 1 nmol ¹⁴CO₂/h.

^c The total cellular protein content was 460 pg.

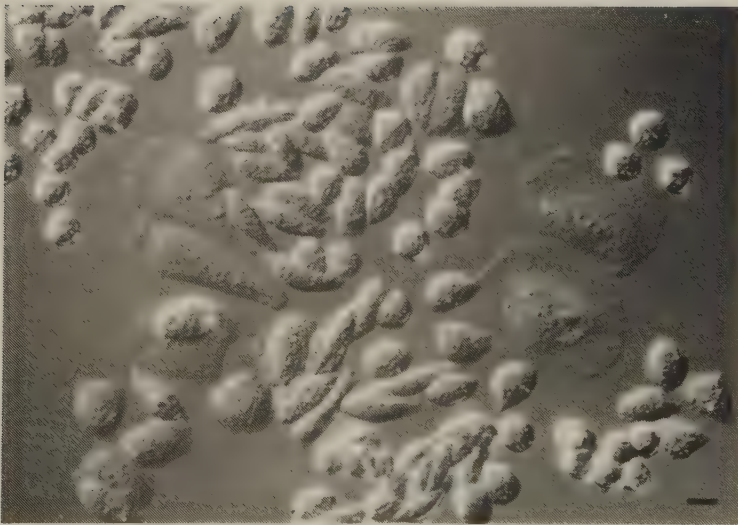


FIGURE 1. Nomarski differential interference contrast micrograph of growing (non-fixed) CDC overproducing mutant CHO cells. In addition to the numerically dominating small and spheroidal cells, some giant and flattened cells are seen. The bar indicates 20 μm .

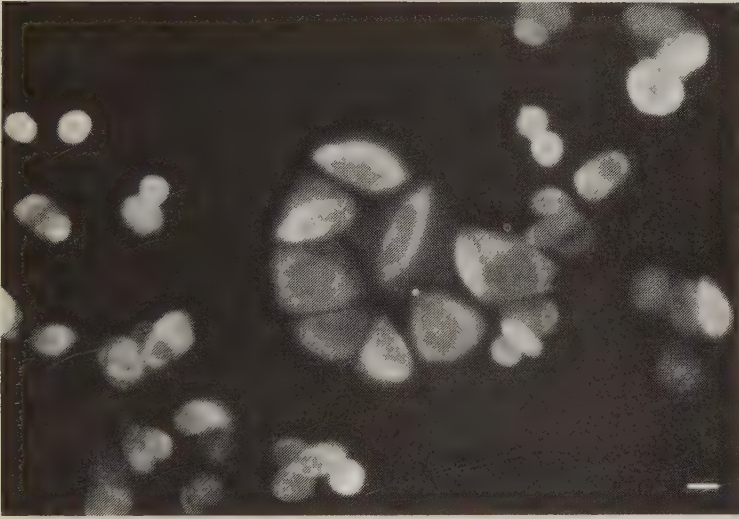


FIGURE 2. Indirect immunofluorescence micrograph of a whole-cell preparation of ODC overproducing mutant CHO cells, showing the localization of ODC protein. Serum-stimulated cells, grown on coverslips, were fixed in 4% paraformaldehyde supplemented with 5 mM dithiothreitol and incubated in permeabilizing buffer containing diluted (1:640) rabbit anti-serum raised against homogeneous mouse kidney ODC. The site of the antigen-antibody reaction was revealed by FITC-conjugated goat anti-rabbit IgG using a 1:20 dilution. The nucleus exhibits less intense fluorescence than the cytoplasm, in small as well as large cells. The high fluorescence intensity seen in the perinuclear region is a consequence of the spatial distribution of the nucleus versus the cytoplasm in cells attached to a substratum, i.e. most of their cytoplasm is simply contained in the perinuclear region. The bar indicates 20 μ m.

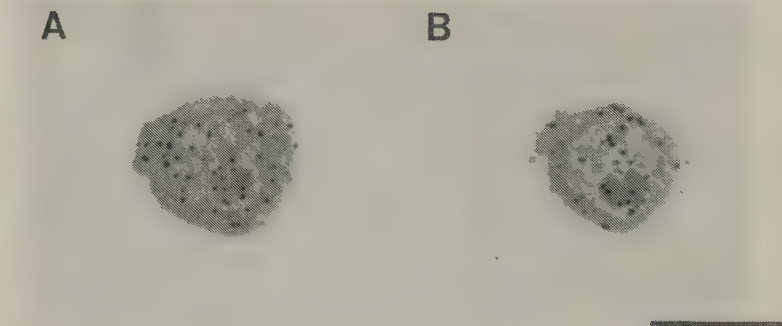


FIGURE 3. Light microscope autoradiographic localization of ^3H -DFMO-bound ODC in $1\ \mu\text{m}$ sections of ODC overproducing mutant CHO cells. Serum-stimulated cells were pulse-labeled (4 h) with ^3H -DFMO, and subjected to a chase with unlabeled DFMO (10 mM, 60 min). The cells were fixed in 2% glutaraldehyde in cacodylate buffer, osmified, dehydrated, stained (1% phosphotungstic acid and 0.5% uranyl acetate in 90% ethanol), embedded in Epon 812 and sectioned. The $1\ \mu\text{m}$ sections were coated with Ilford K 2 liquid nuclear emulsion and exposed for 2 weeks at 4°C . Following development, the sections were stained in a solution of Richardson's azure II and methylene blue. Silver grains were evenly distributed in the cells. The fact that there was a certain degree of variation even within a cell is indicated by two serial sections (A and B) of the same cell. The number and distribution of silver grains in the cells were largely unaffected by the chase time (0 to 60 min). The bar represents $20\ \mu\text{m}$.

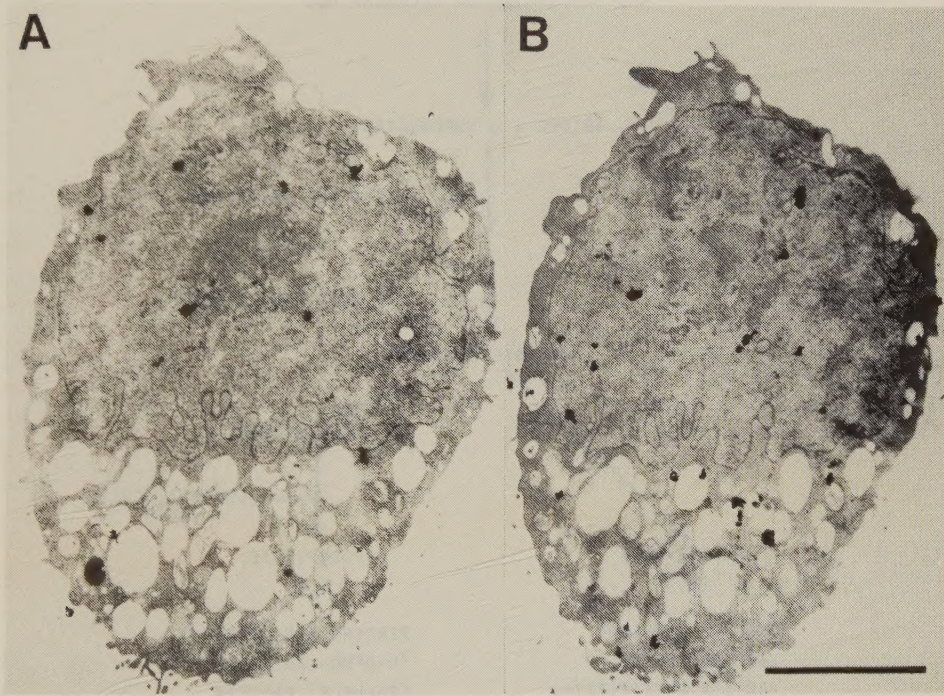


FIGURE 4. Transmission electron microscope autoradiographic localization of ³H-DFMO-bound ODC in ultra-thin sections of ODC overproducing mutant CHO cells. The experimental procedure was the same as that described in Fig. 3, with the exception that the sections were coated with Ilford L 4 liquid nuclear emulsion and exposed for 4 weeks. Following development, the sections were examined in a Jeol 100 CX electron microscope. The distribution of silver grains in the cells coincided with that observed at a light microscopic level in 1 μ m sections. The fact that there was a certain degree of variation even within a cell is indicated by two serial sections (A and B) of the same cell. The number and distribution of silver grains in the cells were largely unaffected by the chase time (0 to 60 min). The bar indicates 5 μ m.

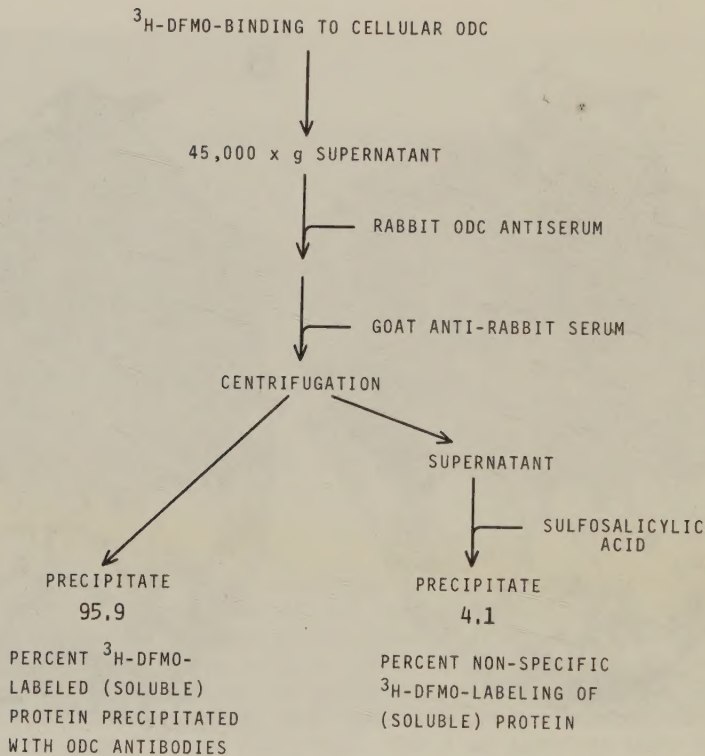


FIGURE 5. Immunoprecipitation of ^3H -DFMO-bound ODC in extracts of ODC overproducing mutant CHO cells. Serum-stimulated cells were pulse-labeled (4 h) with ^3H -DFMO and subjected to a chase with unlabeled DFMO (10 mM; 0, 30 and 60 min). The ^3H -DFMO-labeled cells were sonicated and processed as shown. The rabbit ODC antiserum and the goat anti-rabbit serum were used at a 1:80 and at a 1:1 dilution, respectively. The immunoprecipitate was sedimented by centrifugation (3,000 $\times g$, 30 min) and dissolved in Soluene 350. The supernatant proteins were precipitated and washed in 4% sulfosalicylic acid, and finally dissolved in Soluene 350. Radioactivities were determined by liquid scintillation counting.

